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Induced polyploidy and broad variation in phytochemical traits and altered gene expression in *Salvia multicaulis*

Mansoureh Tavan [a], Ali Azizi [a,*], Hassan Sarikhani [a], Mohammad Hossein Mirjalili [b], Maria Manuela Rigano [c,*]

[a] Department of Horticultural Science, Faculty of Agriculture, Bu Ali Sina University, 65174, Hamedan, Iran
[b] Department of Agriculture, Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, 1983969411, Tehran, Iran
[c] Department of Agricultural Sciences, University of Naples Federico II, Portici, 80055 Naples, Italy

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ABSTRACT

Induced polyploidy is nowadays an important strategy in plant breeding and for the development of new crops. *Salvia multicaulis* Vahl is a valuable medicinal plant that produce precious bioactive metabolites including triterpenic acids (TAs) and phenolic compounds. Hence, at first, for selecting elite lines, both HPLC and GC-MC analyses were performed on fourteen *S. multicaulis* lines. Then, seeds of selected lines of *S. multicaulis* were exposed to different concentrations (0.00, 0.05, 0.1, and 0.2 %) of colchicine for 24 or 48 h. The flow cytometric analysis and chromosome counting were used to confirm ploidy level of tetraploid control ($2n = 4x = 28$, 2C DNA = 1.36 pg) and hexaploid ($2n = 6x = 42$, 2C DNA = 1.97 pg) plants after seven-month. For the first time, the effects of *in vitro* polyploidization on morphological characteristics, TAs and phenolic acid contents as well as on the expression of six TAs biosynthesis related genes were investigated. The highest efficiency of hexaploidy (12.76 %) was achieved 48 h after exposure to 0.1 % colchicine concentration. The hexaploid plants showed different growth traits compared with those of tetraploid control plant; indeed, hexaploid plants had leaves with a darker green color, a lower trichome density, and lower plant height and root length. Moreover, there was a significant increase in rosmarinic acid and caffeic acid content in hexaploid plants compared with tetraploid control plants. Also, the increase of oleanolic acid (1.33 fold) content in hexaploids was associated with a significant increased expression of squalene synthase (*SQS*) and β-amyrin synthase (*BAS*) genes in hexaploid plants. Nevertheless, a significant decreased expression of squalene epoxidase (*SQE*), mixed-function amyrin synthase (*MFAS*), and lupeol synthase (*LUS*) was observed in hexaploid plants, that led to a reduced content of ursolic acid and betulinic acid compared with tetraploid control plants. These results confirmed that polyploidization is a breeding method with stochastic results in secondary metabolites production and gene expression related to biosynthetic pathways.

Abbreviations

TAs	triterpenic acids
UA	ursolic acid
BA	betulinic acid
OA	oleanolic acid
FDS	farnesyl pyrophosphate synthase
SQS	squalene synthase
SQE	squalene epoxidase
LUS	lupeol synthase
MFAS	mixed-function amyrin synthase
BAS	β-amyrin synthase
MS	Murashige and Skoog
HPLC	high performance liquid chromatography
FCM	flow cytometry.

1. Introduction

Artificial polyploidy is the doubling of chromosomes number per nucleus in a cell that is derived either from the same species

* Corresponding authors.
E-mail addresses: mansuretavan@yahoo.com (M. Tavan), azizi@basu.ac.ir (A. Azizi), sarikhani@basu.ac.ir (H. Sarikhani), m-mirjalili@sbu.ac.ir (M.H. Mirjalili), mrigano@unina.it (M.M. Rigano).

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(autopolyploidy) or different species (allopolyploidy) (Stebbins, 1947; Venial et al., 2020). It has been proved that *in vitro* polyploidy is as an appropriate technique to increase the efficiency of medicinal plants through improving bioactive secondary metabolites content, biomass, and adaptability to abiotic stress (Pan-pan et al., 2018; Javadian et al., 2017; Eng and Ho, 2019). The effects of polyploidy on gene expression and gene regulation can be used in plant breeding for the development of novel crops (Dhawan and Lavania, 1996). In fact, induced polyploidy can significantly affect the expression of duplicated genes and can lead to silencing and decreased or increased gene expression with the start of polyploidy or after several generations (Sattler et al., 2016; Zhang et al., 2018). Moreover, artificial polyploidy may increase secondary metabolites production in medicinal plants due to the increase of biosynthetic genes expression (Salma et al., 2017; Salma et al., 2018; Wei et al., 2018; Zhou et al., 2020).

Secondary products, such as terpenoids, phenols, and alkaloids, are accumulated by the plant system under specific conditions (genotype, climate, and edaphic factors). These secondary compounds play a critical role in the adaptation, environmental interaction and resistance to biotic and abiotic stress in plants. They are often studied due to their effects as antifungal, antimicrobial, insecticidal, and antioxidant compounds (Osbourn et al., 2011; Moses et al., 2013; Biswas and Dwivedi, 2019). Polyphenols, including flavonoids, phenolic acids, and tannic acids, have significant anti-oxidation, anti-inflammation and anti-bacterial properties, as well as strong enzyme inhibition, glycation inhibition, and miRNA interference effects (Fraga et al., 2010; Xie and Chen, 2013; Xie et al., 2014; Tavan et al., 2020). Terpenoids are also the most abundant and diverse natural compounds produced in many plants and include mono, di, tri, sesqui, and sesterterpenoids.

Triterpenic acids (TA) including ursolic acid (3β-hydroxyurs-12-en-28-oic acid, UA), oleanolic acid (3β-hydroxyolean-12-en-28-oic acid, OA), and betulinic acid (3β-hydroxylup-20(29)-en-28-oic acid, BA) related to the ursane, oleanane, and lupane backbones, respectively, have various biological activities (Mitaine-Offer et al., 2002; Liu et al., 2005; Somova et al., 2003). UA and OA exhibit anti-inflammatory, antimicrobial, hypoglycemic, and hepatoprotective activity (Sohn et al., 1995; Misra and Laatsch, 2000), while BA exhibits HIV inhibitory activity, antimalarial activity, and antitumor activity (Pisha et al., 1995; Bringmann et al., 1997). Furthermore, the effects of UA, OA, and BA for the improvement of type 2 diabetes have been recently proved (Silva et al., 2016). These three important TA, in addition to the *Salvia* genus, were isolated and identified from other genera of the Lamiaceae family, including *Dracocephalum moldavica, Lophanthus anisatus, Satureja hortensis* (Shanaida et al., 2018), *Rosmarinus officinalis* (Razboršek et al., 2008), *Thymus persicus* (Bakhtiar et al., 2014), *Ocimum Species* (Pai and Joshi, 2017), and *Melissa officinalis* (Jäger et al., 2009).

Terpenoids are a large group of secondary metabolites derived from the isoprene unit (C5), isopentyl diphosphates (IPP) and its isomer dimethylallyl diphosphate (DMADP). These components are condensed sequentially by prenyltransferases and prenyl diphosphates and are formed through the cytoplasmic mevalonic acid (MVA) and the plastidial methylerythritol (MEP) pathways. The triterpenoids (30C) biosynthesis begins with the production of squalene and oxidosqualene precursors. 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, and these mechanisms entail key cation intermediates such as dammarenyl, baccharenyl, and lupenyl cations. Finally, three valuable triterpen acids of UA, OA, and BA, are produced by cytochrome P450s (CYP450s) enzymes (Biswas and Dwivedi, 2019; Haralampidis et al., 2002; An et al., 2020) (Fig. 1). CYP450s are involved in catalyzing the sequential steps in the biosynthetic pathways of secondary metabolites. Indeed, CYP716A155, CYP716A252, and CYP716A253 were identified as converting lupeol, α-amyrin and β-amyrin into BA (in rosemary), UA and OA (in sweet basil), respectively (Misra et al., 2014; Misra et al., 2017; Huang et al., 2019). So far, some key genes in the biosynthetic pathway of TAs were characterized from several important Lamiaceae species, including

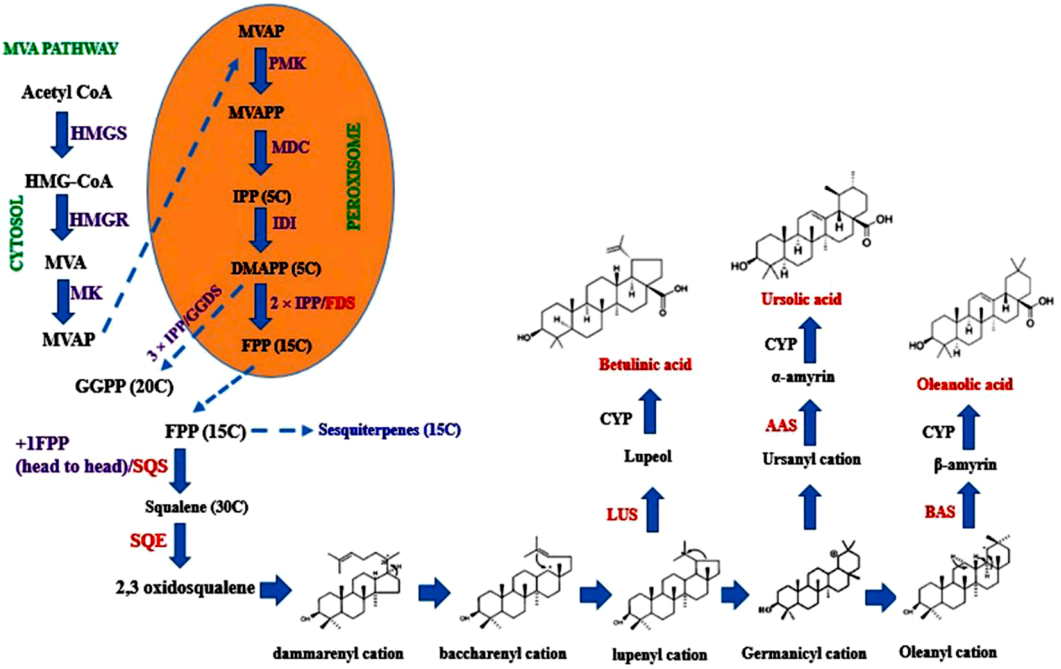

Fig. 1. The biosynthetic pathway of triterpenic acids of ursolic, betulinic, and oleanolic acids.
MVA: mevalonic acid, HMGS: 3-hydroxy-3-methylglutaryl-CoA synthase, HMG-CoA: 3-hydroxy-3-methylglutaryl-CoA, HMGR: 3-hydroxy-3-methylglutaryl-CoA reductase, MK: Mevalonate kinase, MVAP: Mevalonate-5-phosphate, PMK: Phosphomevalonate kinase, MVAPP: Mevalonatediphosphate, MDC: Mevalonate-5-decarboxylase, IPP: Isopentenyl diphosphate, IDI: Isopentenyl-diphosphate isomerase, DMAPP: Dimethylally diphosphate, FDS: Farnesyl pyrophosphate synthase, FPP: Farnesyl diphosphate, GGDS: geranyl geranyl diphosphate synthase, GGPP: geranyl geranyl diphosphate, SQS: squalene synthase, SQE: squalene monooxygenase or epoxidase, LUS: lupeol synthase, CYP: cytochrome P450, AAS: α-amyrin synthase, BAS: β-amyrin synthase.

Ocimum basilicum (Misra et al., 2014), *Salvia officinalis* L. (Ali et al., 2017), *Salvia guaranitica* (Ali et al., 2018), *Rosmarinus officinalis*, and *Thymus persicus* (Aminfar et al., 2019). Starting from these data, additional genes in other species with unknown function can be associated with biological processes by co-expression gene networks (Li et al., 2015; Serin et al., 2016; Ma et al., 2018). Transcriptomic analyses can also be performed to evaluate transcript levels in different conditions and specific tissues and get further insights into TAs metabolic pathways (Aminfar et al., 2019).

The *Salvia* genus is one of the largest genera of the mint family (Lamiaceae) that is widespread throughout the world; however, only one species exist in Australia and no species can be found in New Zealand (Drew et al., 2017). Some species are cultivated worldwide and some species grow as endemic only in certain areas (Sharifi-Rad et al., 2018). There are fifty-eight species of *Salvia* in Iran, of which seventeen species are endemic to this region (Rajabi et al., 2014). The *Salvia* genus has been recognized as a valuable medicinal plant and as an important source of bioactive compounds, such as terpenoids and phenolics (Dweck, 2000; Ulubelen, 2000). Also, the chemical composition of essential oil and the various biological activities of extracts from different species of *Salvia* have been studied (Rajabi et al., 2014). In addition, Jash et al. (Jash et al., 2016) identified about 214 triterpenoids from 113 *Salvia* species as well as the bioactive properties of 111 terpenoids (di, tri, sesqui, and sesterterpenoids) have been identified in this genus (Ulubelen, 2003).

S. multicaulis Vahl is an important species of the *Salvia* genus that contain important secondary metabolites, including phenolic acids, TAs and essential oil (Ali et al., 2017; Gharenaghadeh et al., 2017; Abdollahi-Ghehi et al., 2019; Tavan et al., 2020). Also, this plant is an endemic species growing in the Middle East region and as a wild plant in different parts of Iran (Ulubelen et al., 1997). The present study was performed in three steps. Initially, the screening of fourteen *S. multicaulis* lines in terms of TAs and essential oil compounds was performed. Then, *in vitro*-induced polyploidy of selected lines of *S. multicaulis* was carried out. The third step included the analyses by real-time quantitative PCR (qRT-PCR) of the expression of six genes coding for triterpene synthases including farnesyl pyrophosphate synthase (*FDS*), squalene synthase (*SQS*), squalene epoxidase (*SQE*), *LUS, BAS*, and mixed-function amyrin synthase (*MFAS*), involved in the biosynthesis of BA, OA, and UA triterpenoids, in the lines with different ploidy levels of this *Salvia* species. Finally, the combination of data from qRT-PCR and HPLC allowed us to get further insights into the relationship between transcripts levels and TAs content in the polyploid lines.

2. Materials and methods

2.1. Plant material

The aerial parts and seeds of fourteen *S. multicaulis* lines (*SMPs*) were collected randomly (for each line, nine plants in three replicates every five meters) from the north-west to the south-west of Iran in June 2018 (Table S1). A voucher specimen of *S. multicaulis* (Number: A-Hort-97-SMP) are stored within the herbarium of Bu-Ali-Sina University, Hamedan, Iran.

2.2. In vitro induction of polyploidy

The seeds (30 seeds for each treatment) of *SMP1* (*S. multicaulis*, Taleghan population) were *in vitro* disinfected according to Ejtahed et al. (2015). Subsequently, they were *in vitro* treated with filter-sterilized colchicine solution (0.0, 0.05, 0.1, and 0.2 % (w/v) concentrations) for 24 and 48 h on an orbital shaker (Heidolph, Germany, 100 rpm) at 25°C under dark, and then washed 3 times with autoclaved dH2O. Then, excised embryos of *S. multicaulis*, due to low germination rate, were grown on MS medium with 5% sucrose and supplemented with 0.5 mg / l GA3.

The seedlings were cultured in shoot multiplication medium (MS medium with 0.5 mg/l BAP and 0.1 mg/l IAA). After 30 days, the explants for root proliferation were transferred to MS medium with 1 mg/l IBA growth regulator. The plants that survived were monthly subcultivated on MS medium free of growth regulators and were kept under 16/8 h (light/dark) conditions.

At interval of seven months, the plants were used for flow cytometric analysis, the chromosome counting, morphological and anatomical traits as well as phytochemical analysis and the investigation of genes expression .

2.3. Flow cytometric analysis and chromosome counting

For the DNA ploidy level estimation, almost 25 mg leaf tissue from the regenerated plants of *S. multicaulis* along with the same mass of *Glycine max* L. (as an internal standard with 2C = 2.50 pg of DNA) were chopped and stained for flow cytometric (FCM) analysis according to 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, 2003).

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

2.4. Stomata and morphological characterization of tetraploid control and hexaploid 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 plant weight were compared between tetraploid control and hexaploid 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).

2.5. Extraction and HPLC analysis

The dried and powdered aerial parts of *S. multicaulis*, 0.5 g for each one of the tetraploid control and hexaploid plants as well as *SMPs*, were prepared. Then extraction and high performance liquid chromatography (HPLC) analysis for phenolic compounds of rosmarinic acid, salvianolic acid A, chlorogenic acid, caffeic acid, 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). In addition, these compounds were recognized by comparing their retention times with those of the standards. Also, content of compounds was determined using their standards calibration curve.

2.6. Essential oil isolation and analysis procedure

Dried aerial parts (10 g) of the *SMPs* plants were powdered and the oil isolated using a micro-Clevenger-type apparatus. Gas chromatography–flame ionization detector (GC–FID) and GC–mass spectrometry (GC–MS) analyses as well as identification and quantification of the oils components were carried out as described previously (Raeisi et al., 2015).

2.7. Total RNA extraction and gene expression analyses

Total RNA was extracted from *in vitro* explants of *S. multicaulis* 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 synthesize 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 S2). qRT-PCR was performed to determine the expression of six genes encoding TAs biosynthetic enzymes in different ploidy levels of *S. multicaulis* 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 (D'Agostino et al., 2019).

2.8. Statistical analysis

The statistical analysis was performed with SAS Ver. 9.1 (Cary, NC, USA) software. Means were compared using Duncan's multiple range tests at $p < 0.05$.

3. Results

3.1. The screening of populations and in vitro polyploidy induction

Both the HPLC and GC-MC analyses were performed for selecting the elite lines of *SMPs*. The results indicated that *SMP1* (*S. multicaulis*, population of Taleghan) is the best population in term of contents of TAs (7.98 ±1.19, 5.95 ±0.79, and 6.39±0.48 mg/g DW for UA, BA, and OA, respectively) (Table S3) and the major constituents of the essential oil (32.37, 23.20, 7.74, and 7.19 % for 1,8-Cineole, α-Pinene, Camphene, and Camphor, respectively) (Table S4).

Then, the seeds of *SMP1* (as the selected population) were treated with three concentrations (0.05, 0.1, and 0.2% w/v) of colchicine for 24 or 48 h and were grown on MS medium as described in Materials and Methods. The highest mortality rate was demonstrated at a concentration of 0.2% colchicine, after one month. After plants proliferation, they were subcultured monthly on MS medium without growth regulators. FCM analysis was used to confirm ploidy level of plants after seven-month. After subculturing of treated plants, out of the 47 plants survived, 12.76% hexaploid plants were obtained (Table 1). The most effective treatment for polyploidy induction was 0.1% colchicine concentration and 48 hours treatment duration.

In FCM analysis, the standard plant peak was set in the 92.25 channel. The peak of *S. multicaulis* tetraploid control plants was located on two channels (50, and 80; 2C and 4C, respectively) while the peak of the hexaploid plants (6x) was only placed in the channel 70-75 (2C) (Fig. 2A and B). Mean 2C DNA content was obtained for tetraploid and hexaploid plants of *S. multicaulis* (1.36 ± 0.03 and 1.97 ± 0.05 pg, respectively).

Table 1
The number of regenerated tetraploid control and hexaploid plants of *S. multicaulis*.

Colchicine concentration (%)	Exposure time (h)	No. of treated seeds	Germination (%)	No. Ploidy levels of treated plants	
				4*x*	6*x*
0	24	30	46.7	20	0
	48	30	40.0	17	0
0.05	24	30	30.0	14	0
	48	30	23.3	12	0
0.1	24	30	20.0	10	0
	48	30	16.7	5	6
0.2	24	30	0.0	0	0
	48	30	0.0	0	0

Also, flow cytometry results of the tetraploid control and hexaploid plants were confirmed by chromosome counting. The results showed that the chromosome number of the *in vitro* natural controls was $2n = 4x = 28$ (Fig. 3A) while the chromosome number of treated plants was $2n = 6x = 42$ (Fig. 3B). Chromosome number 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 were determined in diverse cytological studies on the genus *Salvia* and the ancestral chromosome base number seems to be $x = 7$ (Ranjbar et al., 2015).

3.2. Stomata and morphological characterization of tetraploid control and hexaploid plants

After the ploidy levels were confirmed, the morphological and anatomical characteristics of tetraploid control and hexaploid plants were investigated. In terms of appearance, hexaploid plants of *S. multicaulis* had leaves with a darker green color that were thicker and had a lower trichome density (Fig. 4). Also, colchicine treatment delayed root growth and resulted in reduced plant growth. Therefore, in tetraploid control plants, plant height and root length were higher than hexaploid plants; however, the number of leaves in hexaploid plants was higher than tetraploid control plants (Table 2; Fig. 4). In hexaploid plants of *S. multicaulis* the dry and fresh weight was higher than tetraploid control plants (Table 2), perhaps due to higher number of leaves and the greater thickness of leaves and stems in polyploids (Tavan et al., 2015).

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 cell were observed in hexaploid plants compared with tetraploid control plants (Table 2; Fig. 5).

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

There was a significant difference in the content of TAs between tetraploid control and hexaploid plants of *S. multicaulis* ($P \leq 0.05$) (Table 2). The contents of UA and BA in hexaploid plants (0.86 and 0.72 fold, respectively) decreased compared with the tetraploid control plants; whereas, hexaploid plants showed an increase in the content of OA (1.33 fold) compared with tetraploid control plants. While the contents of phenolic acids of rosmarinic acid and caffeic acid (1.51 and 1.50 fold, respectively) were increased in hexaploid plants compared to tetraploid controls, there was no significant difference in the contents of other phenolic acids (Table 2).

3.4. Expression analysis of TAs biosynthetic genes in plants with different ploidy levels

As shown in Fig. 1, three enzymes of *FDS, SQS*, and *SQE* at the beginning of the pathway and three enzymes of *MFAS, LUS*, and *BAS* at the end of the biosynthetic pathway play an important role in the biosynthesis of TAs (UA, BA, and OA). qRT-PCR technique quantitatively measures changes in gene expression and it is the only rapid, sensitive, and accurate system that can assess a few copies of mRNA (Valasek and Repa, 2005). It was used to demonstrate the relationship of UA, BA, and OA content with the expression pattern of key genes in the biosynthetic pathway of these TAs and in lines with different ploidy levels of *S. multicaulis*. Therefore, the gene expression levels in the hexaploid plants were compared with those of the tetraploid control

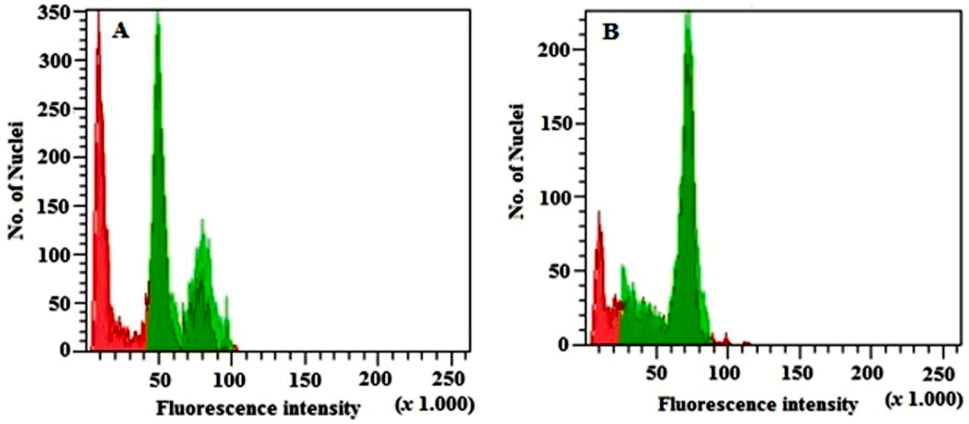

Fig. 2. Histograms of flow cytometric analysis of tetraploid control (A), hexaploid (B) plants of *S. multicaulis*.

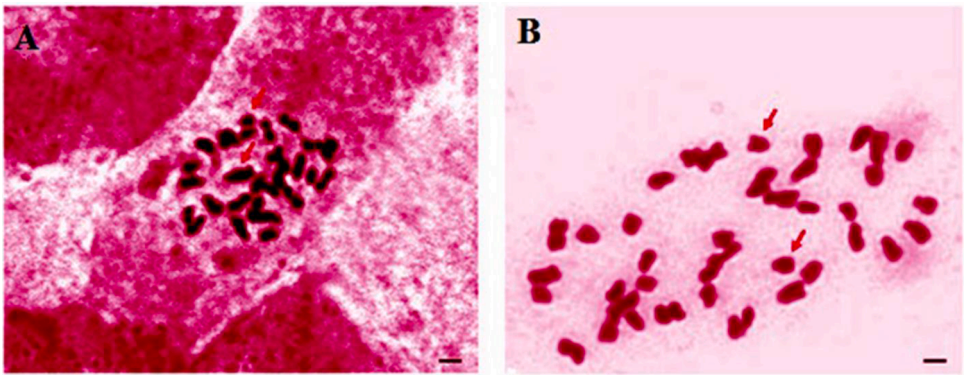

Fig. 3. Chromosome numbers of root tip cells from tetraploid control plants $2n = 4x = 28$ (A); and hexaploid plants $2n = 6x = 42$ (B) of *S. multicaulis*. Bars: 5 μm

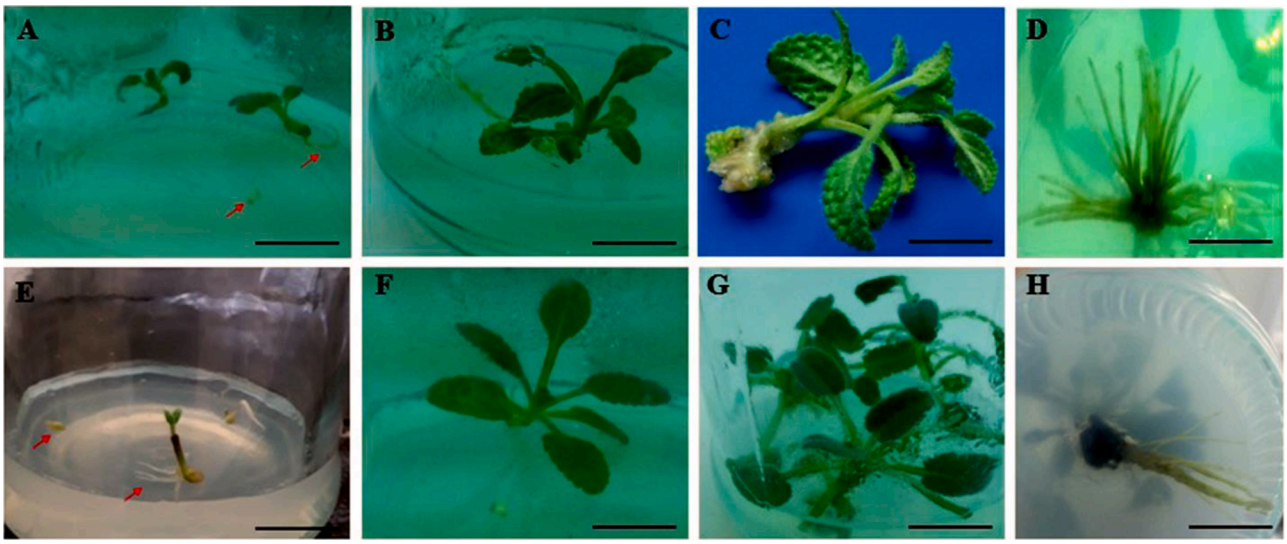

Fig. 4. Comparison of morphological characteristics of *in vitro* plants of tetraploid control (A-D), and hexaploid (E-G), of *S. multicaulis*. Bars = 1cm
A and E: The embryo growth and development of the radicle and cotyledon in the tetraploid control (A) and hexaploid (E) plants. B and F: The early growth and development of true leaves in the tetraploid control (B) and hexaploid (F) plants. C and G: The leaves color and thickness in the tetraploid control (C) and hexaploid (G) plants. D and H: The root growth rate and length in the tetraploid control (D) and hexaploid (H) plants.

counterparts for all studied genes (Fig. 6). The higher expression of *SQS* (4.29 fold) and *BAS* (1.53 fold) genes was observed in hexaploid plants compared with those of tetraploid controls (Fig. 6).

4. Discussion

Induced polyploidy can facilitate plant breeding programs including causing genetic changes in gene expression and gene function and resulting in the improvement of the quantity and quality of secondary

Table 2
Effect of ploidy level on the morphological and stomata characteristics, and the comparison of triterpenic acids and phenolics contents of *S. multicaulis* plants.

6*x*	4*x*	Characteristics
30.6 ± 2.06[a]	24.4 ± 0.84[b]	Length of guard cells (μm)
24.6 ± 0.97[a]	20 ± 0.67[b]	Width of guard cells (μm)
18.4 ± 1.07[b]	22.2 ± 1.99[a]	Stomatal density (μm)
3.35 ± 0.34[b]	4.2 ± 0.24[a]	Plant height (μm)
22.4 ± 1.49[a]	20 ± 1.33[b]	Leaf (No.)
6.3 ± 0.95[b]	9.5 ± 1.08[a]	Root length (cm)
362.5 ± 33.85[a]	300 ± 40.82[b]	Plant dry weight (mg)
1450 ± 135.40[a]	1200 ± 163.30[b]	Plant fresh weight (mg)
6.31±0.18[b]	7.35±0.22[a]	Ursolic acid (mg/g DW)
3.06±0.18[b]	4.25±0.10[a]	Betulinic acid (mg/g DW)
5.84±0.36[a]	4.40±0.16[b]	Oleanolic acid (mg/g DW)
4.75±0.27[a]	3.15±0.12[b]	Rosmarinic acid (mg/g DW)
0.56±0.10[a]	0.38±0.11[a]	Salvianolic acid A (mg/g DW)
0.21±0.03[a]	0.25±0.03[a]	Chlorogenic acid (mg/g DW)
0.30±0.03[a]	0.20±0.05[b]	Caffeic acid (mg/g DW)
0.12±0.05[a]	0.15±0.03[a]	Quercetin (mg/g DW)

The data (mean ± standard deviation) were evaluated from 10 plants each of tetraploid controls and hexaploids. Means were compared using Duncan's multiple range tests and means within each row with different letters (a and b) are significantly different at $p \leq 0.05$.

metabolites. However, the effects of polyploidy are often unpredictable and may significantly vary between species (Touchell et al., 2020). Therefore, in this study, the effect of polyploidization on morphological and anatomical traits as well as phytochemical analysis and expression of the genes of the biosynthetic pathway of TAs were investigated.

The first part of the experiments was performed to screen 14 *SMPs* (as an endemic species in Iran) in terms of the content of TAs and essential oil compounds. There are several studies about bioactive TAs (Topçu, 2006; Abdollahi-Ghehi et al., 2019) as well as essential oil constituents (Ghorbani and Esmaeilizadeh, 2017; Ali et al., 2017) from *Salvia* species including *S. multicaulis*. Also, the investigation of phenolics diversity and antimicrobial and antioxidant activity among 19 wild populations of *S. multicaulis* have been performed recently in our laboratories (Tavan et al., 2020). Abdollahi-Ghehi et al. (2019) reported that the amount of TAs in 22 *Salvia* species native to Iran ranged from 0.06-1.96, 0.17-3.12, and 0.08-4.34 mg/g DW for OA, BA, and UA, respectively. The TAs of BA and OA had the maximum content in the aerial parts of *S. multicaulis* compared with other species of *Salvia*, and the higher amount of UA was observed in *S. officinalis* and *S. multicaulis* (4.34 ± 0.1 and 3.71 ± 0.08 mg/g DW, respectively). The present study indicated that UA is the most abundant compound in all studied population and this result well correlate with other studies on Lamiaceae species (Jäger et al., 2009; Shanaida et al., 2018). Also, our results on major constituents of the essential oils of *SMPs* were in agreement with some previous reports from *S. multicaulis* (Mohammadhosseini et al., 2008; Gharenaghadeh et al., 2017). It should be noted that determination of genetic and phytochemical diversity among wild populations of medicinal plants is an important and basic step for performing breeding programs (Abdollahi-Ghehi et al., 2019). Nevertheless, the different results obtained in the amount of TAs and essential oils of *SMPs* can be affected by the different growth conditions including climatic changes, geographic origins, and genetic variation.

Then, *in vitro*-induced polyploidy was applied to *S. multicaulis* for the first time and hexaploid plants of *S. multicaulis* were obtained. Generally, morphological and anatomical changes are reliable indicators to confirm polyploidy in plants, including changes in leaves shape and color, leaf and stem thickness, trichomes density, root length, plant height, and number of shoots as well as length and width of the stomata guard cell and stomatal density (Pan-pan et al., 2018; Mo et al., 2020). In our study, the growth of hexaploid plants was slower due to delayed root growth compared to tetraploid control plants. Moreover, the number of leaves in hexaploid plants was higher and a lower stomata density along with greater length and width of the stomata guard cell were observed in hexaploid plants compared with tetraploid control plants.

There was no significant difference in the contents of phenolic acids between tetraploid control and hexaploid plants except for rosmarinic acid and caffeic acid. Also, regarding the TAs assessed in hexaploid

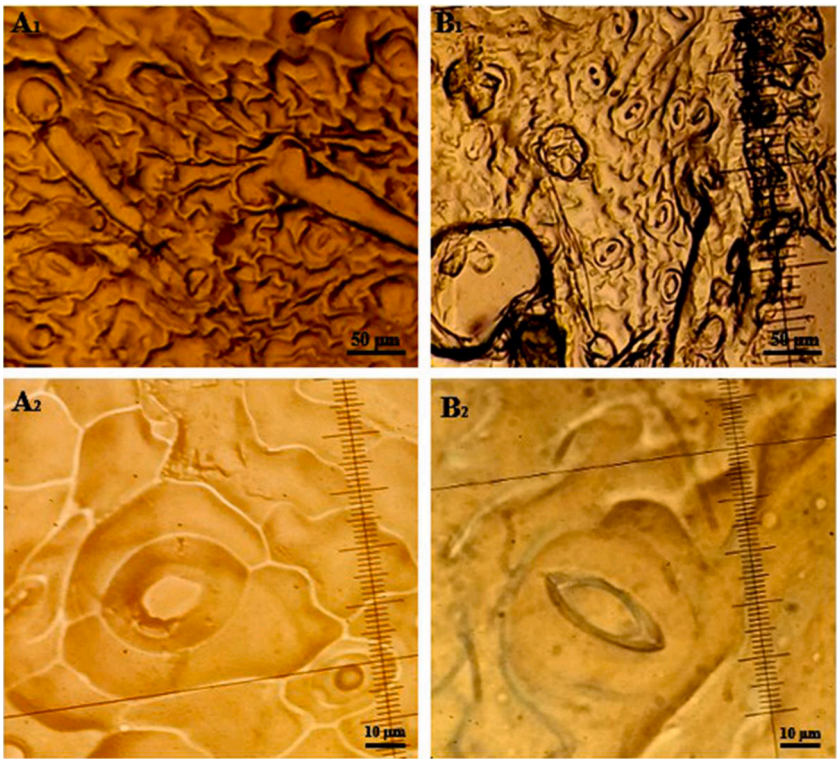

Fig. 5. The observation of stomata characteristics *in vitro* plants including length and width of the stomata guard cell and stomatal density in the tetraploid control (A1 and 2) and hexaploid (B1 and 2) plants of *S. multicaulis*. Bars = 10 and 50 μm

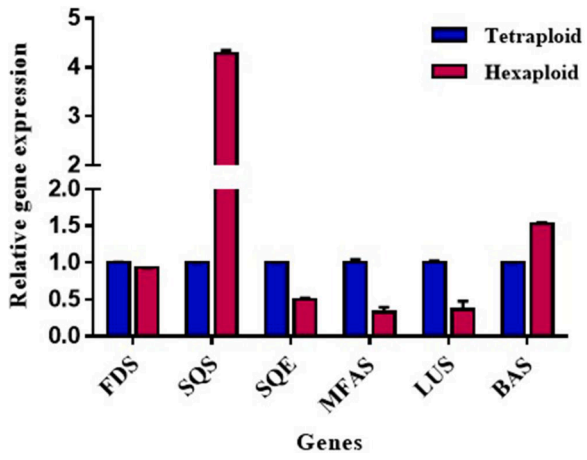

Fig. 6. Relative expression of six genes related to triterpenic acids biosynthetic including farnesyl pyrophosphate synthase (*FDS*), squalene synthase (*SQS*), squalene epoxidases (*SQE*), lupeol synthase (*LUS*), β-amyrin synthase (*BAS*), and mixed function amyrin synthase (*MFAS*) genes in the ploidy levels of tetraploid control and hexaploid plants of *S. multicaulis*. Error bars are shown as standard deviation (n = 3).

plants and compared with those of the tetraploid control counterparts, it was found that OA content of hexaploids plants was significantly higher than tetraploid control plants. On the contrary, contents of BA and UA in tetraploid control plants of *S. multicaulis* were remarkably higher than those of hexaploids. Therefore, increased ploidy levels may not always be associated with the enhancement of the content of secondary metabolites (Lavania, 2005). For example, tetraploids of *Digitalis purpurea, Trigonella foenum-graecum*, and *Mentha spiculata* have reduced content of several metabolites compared with those of diploids (Evans and Evans, 2009). Nevertheless, in recent years, artificial polyploidy has been highly regarded as a way for improving the quantity and quality of plant metabolites (Saminathan et al., 2015; Tavan et al., 2015; Dou et al., 2017; Noori et al., 2017; Chung et al., 2017; Julião et al., 2020; Cara et al., 2020). Indeed, these studies express the fact that induced polyploidy is a stochastic source of variation (Aversano et al., 2013).

Finally, differences in the expression patterns of key genes of the biosynthetic pathway of three TAs were analysed in two lines of *S. multicaulis* with different ploidy levels. The highest gene expression difference was related to the *SQS* gene in hexaploid plants compared with tetraploid control plants (Fig. 6). Regarding *OSCs* genes, a higher gene expression of the *BAS* gene was observed in hexaploids compared with tetraploid control plants (Fig. 6). Interestingly, *OSCs* use 2,3-oxidosqualene as a common biosynthetic intermediate for converting it into different pentacyclic triterpenoids including BA, OA, and UA. Also, there are reports that overexpression of *HMGR, FDS*, and *SQS* genes in the biosynthetic pathway of the isoprenoid increases triterpenoids production (Seo et al., 2005; Muñoz-Bertomeu et al., 2007; Kim et al., 2010). However, our results showed that, despite an increased gene expression of *SQS* in hexaploid plants, the expression of the genes *FDS* and *SQE* decreased. Also, gene expression of *OSCs* of *LUS* and *MFAS* decreased, which resulted in reduced production of BA and UA. Nevertheless, increased expression of the *BAS* gene had a positive effect on OA content that increased in hexaploids. Therefore, it can be concluded that overexpression of genes at the beginning of the biosynthetic pathway of triterpenoids in different species and with different ploidy levels can have different effects on the expression of *OSCs* genes as well as on the production of triterpenoids. On other hand, reduced gene expression of *SQE* in hexaploids of *S. multicaulis* at the beginning of the biosynthetic pathway led to reduced gene expression of *OSCs*, except *BAS,* compared with tetraploid control counterparts. Dong et al. (2018) reported that co-expression of three *CpSEs* (squalene epoxidase from *Cucurbita pepo*) with four different triterpene cyclases boost triterpene production. Also, there are reports that demonstrate that plant genomes may contain several *SQE* copies; for instance, the *Arabidopsis* genome has six *SQE* copies although only three can oxidize squalene (Rasbery et al., 2007; Laranjeira et al., 2015). Thus, our hypothesis is that the striking reduction of gene expression of *SQE* in polyploid plants may have led to the reduction of gene expression of *OSCs* and reduced TAs content; although, the transcriptional level is not always straightly connected to the enzymatic activity, as multiple regulatory systems are involved in enzyme activity and efficiency (Vogel and Marcotte, 2012; Aminfar et al., 2019). In general, TAs accumulation may be related to the expression of each of the six genes of the biosynthetic pathway of these compounds. The artificial polyploidy can provide evidence of the incidence of epigenetic and genetic changes as well as boosting variations in gene expression levels without modifying the DNA sequence. Indeed, this phenomenon occurred by modifications in dosage compensation, the chromatin compaction levels, and RNA interference (Osborn et al., 2003; Soltis et al., 2004; Sattler et al., 2016). Therefore, polyploidization could affect gene expression and gene regulation and these effects have been elaborated in a number of polyploid plants such as *Chrysanthemum nankingense* (Dong et al., 2016), *Chrysanthemum lavandulifolium* (Gao et al., 2016), and *Arabidopsis thaliana* (Liu et al., 2017). So far, there has been no report about the effect of induced polyploidy on the expression of genes involved in the biosynthetic pathway of terpenoids. Recently, Tarkesh Esfahani et al. (2021) indicated that the expression level of genes related to thebaine biosynthesis increased in tetraploids compared to those of the diploids of *Papaver bracteatum* L. Also, increased gene expression related to cichoric acid biosynthesis was observed in tetraploids of *Echinacea purpurea* L. compared to diploids (Xu et al., 2014). Nevertheless, the reduction of terpenoids and amino acids contents in some allopolyploid lines of *Solanum tuberosum* compared to diploids was reported (Cara et al., 2020). Therefore, it can be concluded that duplication of genome can increase or decrease production of some secondary metabolites, depending on the analyzed species.

5. Conclusion

In this study, *SMP1* was selected as the best population after screening fourteen *SMPs* in terms of the content of TAs and essential oil compounds. The highest hexaploid induction efficiency (12.76 %) was achieved after 48 hours of exposure to 0.1% colchicine concentration. While there was no significant difference in the contents of phenolic acids between tetraploid control and hexaploid plants except for RA acid and CA, the polyploidization caused an increased content of 1.33 fold of OA and reduced UA and BA contents in hexaploids of *S. multicaulis*. Indeed, the gene expression of *SQS* and *BAS* in hexaploid plants of *S. multicaulis* were upregulated, leading to higher content of OA. Also, polyploidy induction seems to cause a significant reduction in the expression of the gene *SQE*, which play a key role in *OSCs* expression and TAs production. These results showed that increased ploidy level can result in different changes in the transcriptome and metabolome in different species.

6. 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.

Declaration of Competing Interest

The authors confirm that they have no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scienta.2021.110592.

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