Metabolite profiling and molecular responses in a drought-tolerant savory, *Satureja rechingeri* exposed to water deficit

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Abstract

This study aimed to determine the response of *Satureja rechingeri* to water deficit by quantifying the expression of three targeted genes and four traditional reference genes using quantitative real-time PCR analysis (RT-qPCR). Drought stress was imposed by withholding water 4 months after planting. Profiling of volatile and non-volatile compounds using gas chromatography/mass spectrometry (GC/MS) and high-performance thin layer chromatography (HPTLC) showed an increasing–decreasing trend of major phenolic and terpenoid compounds such as rosmarinic and caffeic acids, carvacrole, thymol and p-Cymene. Drought stress also lead to significant increases in oil yield, soluble sugars and proline as well as significant reductions in leaf water potential (LWP), relative water content (RWC), and pigments. Metabolite profiling revealed the strategies savory employed to generate different biochemical phenotypes. RT-qPCR analysis showed that up-regulation of the three genes [1-deoxy-D-xylulose 5-phosphate reductoisomerase (*DXR*), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*HMGR*) and rosmarinic acid synthase: 4-coumaroyl-CoA (*RAS*)] selected from the phenylpropanoid and terpenoid biosynthesis pathways were markedly enhanced at the transcript levels of the regulatory steps and directly increased the production of secondary metabolites, including phenolic and terpenoid compounds. Actin protein (*ACT*), elongation factor 1- α (*EF1 α*), glyceraldehyde-3-phosphate dehydrogenase cytosolic (*GAPC*) and ubiquitin-conjugating enzyme (*UBC*) were used as traditional reference genes. *UBC*'s suitability as the reference genes were verified in *S. rechingeri*. The study's results provide the foundation for gene expression analysis of savory and other species of Lamiaceae. Thus, the effective application of drought stress before harvesting can increase the quantity and quality of raw material.

Keywords *Satureja rechingeri* · Phenylpropanoid biosynthesis pathway · Physiological parameters · RT-qPCR analysis · Drought stress

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Introduction

Savory products are increasingly widespread and include fresh and dried herbs, oleoresins, and essential oils. The essential oils and extracts of savory can be used medicinally in different biotic and abiotic treatments for its antioxidant, antiviral, analgesic, antifungal, disinfectant, antimicrobial, anti-inflammatory, and anticancer properties (Shariat et al. 2013). Rapidly increasing demand for savory products is outpacing what natural populations can supply; therefore, improving cultivation of savory under different conditions, including water deficient ones, will facilitate future production increases while protecting natural biodiversity and climate change (Jaleel et al. 2007). It is well established that various stress conditions, including drought, impact metabolic pathways such as terpenoid and phenylpropanoid, which are responsible for producing

terpenes and phenols in the Lamiaceae family (Shariat et al. 2016).

The production of terpenoids arrange within two distinct biochemical pathways: one in cytoplasm and the other in plastids. These are named the methylerythritol 4-phosphate (MEP) pathway and the mevalonate (MVA) pathway (Figure S1a). These two pathways produce universal precursors for the biosynthesis of terpenoids: isopentenyl diphosphate (IDP) and its allylic isomer, dimethylallyl diphosphate (DMADP). A rate-determining step for the isoprenoid biosynthesis pathway was chosen from the MVA pathway, the gene coding for *HMGR* (EC 1.1.1.88) (Chappell et al. 1995). This step catalyzes the conversion of HMG-CoA into MVA (Goldstein and Brown 1990). The gene coding for *DXR* (EC1.1.1.267) was chosen from the MEP pathway. It is the second committed step in the production of the isoprenoids pathway and plays a critical role in directing the intermediate flux into the *O*-isopropylidene and dimethylallyl diphosphate biosynthetic pathways (Mahmoud and Croteau 2001). In addition, one enzyme involved in the biosynthesis of rosmarinic acid is rosmarinic acid synthase (RAS) (Petersen 1997). RAS is probably the most specific enzyme for rosmarinic acid production in the phenylpropanoid biosynthetic pathway (Weitzel and Petersen 2011) (Figure S1b), but not much is known about this enzyme in *Satureja* species. The precursor for the formation of rosmarinic and caffeic acids is L-phenylalanine; it is formed by the shikimic acid pathway, which also delivers L-tyrosine as well as shikimic and quinic acids. The well-known enzymes of the phenylpropanoid pathway start with the phenylalanine ammonia-lyase transformers, L-phenylalanine to 4-coumaroyl-CoA. RAS, as the characteristic enzyme of rosmarinic acid biosynthesis, transfers the 4-coumaroyl moiety from the intermediary precursors of 4-coumaroyl-CoA to the aliphatic hydroxyl group of 4-hydroxyphenyllactic acid (pHPL) and forms an ester, which is then meta hydroxylated into rosmarinic acid by cytochrome P450 monooxygenase activities at the aromatic positions 3 and 3' (Petersen 1997).

Even though the terpenoid and phenylpropanoid biosynthetic pathways have been characterized in detail at the molecular level, little is known about their regulation during plant responses to drought stress, especially in medicinal plants (Pateraki and Kanellis 2010). At the biochemical level, several studies have demonstrated that the massive increase of monoterpenes and phenolic compounds induced by drought stress is much higher than the corresponding decrease in biomass (Selmar and Kleinwächter 2013). Understanding the expression patterns of the genes involved in metabolite production and their association with different environmental conditions could facilitate the economical production of medicinal plants. To the authors' best knowledge, this is the first study to investigate phenylpropanoid

and terpenoid biosynthesis in response to drought stress in savory species.

The main motivations for this research were to investigate the expression profiles of three genes from the MEP, MVA, and phenylpropanoid pathways in *S. rechingeri* in response to drought stress and to ascertain a suitable reference gene for savory species. Another objective was to study the relationship between quantitative gene expression and the production of secondary metabolites such as carvacrol and thymol. A final objective was the proper use of drought stress to enhance medicinal compounds and assess their effects on several physiological characteristics.

Materials and methods

Plant material, drought stress, and physiological parameters

A number of naturally grown individuals of savory (*S. rechingeri*) were collected from Dehloran regions (located in Ilam province, Iran). The mature plants were recognized by regional floras and authors with taxonomic and floristic reference (Rechinger 1982). Voucher specimen (Code: RIFR 101,598) were located at the Herbarium of Research Institute of Forests and Rangelands of Iran. Since savory is a cross-pollinated plant with a high variety of metabolic characteristics, micro-propagation was utilized to prepare clones from *S. rechingeri*. Among the produced clones, only a clone which was superior for morphological characters selected and transferred to the greenhouse. After adaptation and establishment in a greenhouse, the clone was transferred to 30 kg-pots and regularly irrigated for 4 months. 18 pots, which were superior in appearance, such as height and diameter of the canopy, were selected and divided into six groups of the triad. The first three pots were used as controls and five other groups were used every 3 days after irrigation discontinued. So that the same three plants (biological replicates) used in RT-qPCR and each other experiment. Preliminary and experimental design of the greenhouse pot using a completely randomized design shown in Fig S2. In each pot, five seedlings (clones) were cultivated, because in the process of micro-propagation, five explants were used in a tissue culture glass jar and all content of each one was transferred to a pot. When plants were 4 months at flowering stage, drought stress was applied by discontinuing irrigation for 2 weeks, and several physiological parameters were measured every 3 days. A time-domain reflectometer (HH2 Moisture Meter, Delta T Devices, UK) was used to measure the volumetric soil water content. LWP was measured using the liquid immersion method (Michel 1972). RWC (%) was calculated using the equation described by Boyer (1969). Proline content ($\mu\text{g g}^{-1}$ FW) and total soluble sugars

($\mu\text{g g}^{-1}$ DW) were measured using the methods reported by Bates et al. (1973) and Irigoyen et al. (1992), respectively. Plant pigments were extracted using the acetone method (Linchtenthaler and Welburn 1983). Essential oils were extracted by the water distillation technique (Clevenger) for 3 h. The yield of essential oil was calculated from the percentage of essential oils achieved from 100 g of dry mass.

Quantitative gene expression

Primer design and verification of amplified products

Three targeted genes (*DXR*, *HMGR*, and *RAS*) were selected for RT-qPCR based on previously published researches (Table 1). Four common candidate reference genes (*ELF1- α* , *UBC*, *GAPC*, and *ACT*) were also selected based on reports on other species (Xiao et al. 2014; Wu et al. 2016). Sequences were obtained from the National Center for Biotechnology Information (NCBI, USA), as listed in Table 1. Specific primers of the *DXR* and *HMGR* genes were designed for RT-qPCR analysis using Primer 3 and Oligo-Analyzer 3.1 software. As gene sequence reports for *Satureja* species were not available in the NCBI international databases, the gene sequences of *RAS*, *ELF1- α* , *UBC*, *GAPC*, and *ACT* in other plants were referenced and compared to sequences in other genera of Lamiaceae family using NCBI blast tools. The acquired similar sequences were aligned, and the conserved points in the sequences were identified using the web-based software, ClustalW2. Primers were designed, using the same software as mentioned above. Gene-specific primers are listed in Table 1. PCR was performed on the samples with primers (10 pmol) and Master Mix (Amplicon, Denmark). A 100-bp DNA ladder standard (Fermentas, Canada) was used to calculate the molecular weights of the amplified products. The efficiency of each primer was determined by five-point standard curves of different concentrations from pooled cDNA (each assay contained 50, 10, 2, 0.5, and 0.1 ng of cDNA) in three replications. Each serial dilution was used in a separate real-time reaction, and their threshold cycle values were determined. In a base-10 semi-logarithmic graph, the threshold cycle versus the dilution factor was plotted, and the data conformed to a straight line. The coefficient of determination (R^2) was confirmed to be 0.99 or greater. The calculated reaction efficiency was estimated using Rest 2009 software.

RNA extraction, quality control, and cDNA synthesis

The samples were separated from fresh tissues during drought stress and kept at -80°C . Total RNA was extracted using a GeneAll Ribospin Plant Kit (GeneAll, Seoul, South Korea) and treated extensively with RNase-free DNase I (GeneAll, Seoul, South Korea). The concentration of total

Table 1 Candidate reference genes, primer sequences, and characteristics of PCR amplifications in *S. rechingeri*

Accession no.	Genes	Name	Length (bp)	Primer sequence U/L [5'-3']	Tm ($^\circ\text{C}$)	Slope (-)	E (%)	Mean Ct	SD (+/- Ct)	CV (%)
AB677954	<i>DXR</i>	1-deoxy-D-xylulose 5-phosphate reductoisomerase	172	CAGTGTATCCAAAGGCTTGCC GAGCGTTGCAGAGTCCACT	58.90 60.6	3.352	98.75	22.77	0.96	4.22
AB758241	<i>HMGR</i>	3-hydroxy-3-methylglutaryl-coenzyme A reductase	184	ATGGATGTCATTGGCATTCTGG CGGTAAGGTTCTTGAGCATGTT	59.61 58.93	3.340	99.23	22.78	0.73	3.23
FR670523.1	<i>RAS</i>	Hydroxycinnamoyl-CoA: hydroxyphenyllactate hydroxy cinnamoyltransferase	85	AGACGAAGTCCACATCCC GTGGGAAGAAGATGGCGTT	59.8 59.9	3.331	99.61	22.60	0.59	2.12
D63396	<i>ELF1α</i>	Elongation factor 1- α	267	CTTGCTTTCACCTTGGTGT CTTGGGCTCATGATCTGTT	60.15 60.07	3.334	99.49	18.54	0.12	0.45
KJ1701325	<i>GAPC</i>	Glyceraldehyde-3-phosphate dehydrogenase	198	TCCAAACAGCCTACCAACACA TTGGAATGCAATGCTCTCA	60.15 60.05	3.338	99.3	16.49	0.61	3.69
JN399227	<i>UBC</i>	Ubiquitin-conjugating enzyme	243	CCAAAGGTGCAATTCAGGAC ACTTCTGGGTCCAGCTCCTT	59.69 59.87	3.329	99.7	23.96	0.59	2.47
HM231319	<i>ACT</i>	Actin protein	169	GTGGATTGCCAAAGCAGAAT TAAACGGACAAACCCGGAAG	60.08 59.98	3.343	99.1	16.43	0.7	4.28

RNA was determined using a NanoDrop spectrophotometer (Epoch™ Multi-volume Spectrophotometer System, USA). For semi-quantitative and quantitative expression analysis of *HMGR*, *DXR*, *RAS*, and the four *Satureja* plant reference genes (*ELF1-α*, *UBC*, *GAPC* and *ACT*), total RNA (1 µg) was used to generate first-strand cDNA using a RevertAid First-Strand cDNA Synthesis Kit (Thermo Scientific, USA).

Real-time PCR

Quantitative real-time PCR (RT-qPCR) was performed in a fluorometric thermal cycler (CFX96 Real-Time PCR, Bio Rad, USA) using 5×HOT FIREPol EvaGreen HRM Mix (Solis BioDyne, Estonia) as a fluorescence dye according to the manufacturer's instructions. For the RT-qPCR analysis, RNA was extracted from each of the three individual seedlings at each sampling time (days 0, 3, 6, 9, 12, and 15) as the three biological replicates; three technical replicates were also considered. Reactions were prepared in a 10 µl volume containing the following: 1 µl cDNA template, 0.3 µl of each amplification primer (10 pM), 2 µl EvaGreen HRM Mix with ROX (5X) and 6.4 µl dH₂O. Real-time PCR conditions in the BioRad system comprised a temperature of 95 °C for 15 min for denaturation or primary activation, 40 cycles (each cycle at 95 °C) for 20 s, optimal temperature of primer annealing (57 °C) for 20 s and 72 °C for 30 s, and the final cycle at 60–95 °C for 5 min to obtain the melt curve. The results obtained from the data related to six time points for collecting samples of *S. rechingeri* were analyzed using Rest 2009 software (Pfaffl et al. 2002).

Oil extraction, methanolic extract and chromatography

The shoots of the harvested plants were air-dried in the shade, and both the flower heads and the leaves were exposed to hydrodistillation for 180 min using a Clevenger-type apparatus according to the European Pharmacopeia procedure (Pharmacopée européenne 1997). The obtained oils were dried over anhydrous sodium sulfate and kept in a refrigerator at 4 °C prior to analysis. The content of essential oil (v/w) and yield (%) for each treatment were determined. Gas chromatography-flame ionization detector (GC-FID) and GC/MS analyses were conducted utilizing a Thermoquest gas chromatograph with a flame ionization detector (FID) and a gas chromatograph supplied with a fused silica capillary DB-5 column (ThermoQuest/Finnigan, Manchester, UK). These analyses were carried out following the protocol published earlier (Khadiji-Khub et al. 2014). The extraction procedure for estimating the caffeic, rosmarinic, and carnosic acids was performed according to the method described by Hadian et al. (2010). Preparation of the ursolic acid was performed according to Liang et al.

(2009). A Camag HPTLC system (CAMAG, Muttenz, Switzerland) and TLC aluminum sheets were used according to the method of Hadian et al. (2010).

Statistical analysis

Prior to ANOVA, the normality of data and the homogeneity of variance were tested with IBM's SPSS Statistics 24 software package. A completely randomized design (CRD) was performed with five levels of sampling times. Variables were analyzed by one-way ANOVA ($P \leq 0.05$). To select appropriate reference genes, the data obtained from RT-qPCR were analyzed to study the stability of reference gene expressions and select the best gene using Bestkeeper-1 software (Pfaffl et al. 2002).

Results

Effects of drought stress on physiological parameters

Drought stress had significant effects on the LWP. The average of LWP before inducing stress was -0.6 MPa, but after stopping irrigation, it decreased to -5.8 MPa. Shoot water potential was more negative in drought-stressed plants compared to watered plants across all material ($P < 0.01$). RWC as a proportion of fresh weight was used as a complementary characteristic for LWP. Before imposing stress, *S. rechingeri* had an RWC over 90%, which decreased by around 20% after prolonged drought stress of 15 days. LWP and RWC decreased ($P < 0.01$), while drought stress increased throughout the period water was withheld (Fig. 1). Volumetric soil moisture also decreased as soil water deficit decreased (Fig. 1). A reverse relationship was identified between drought stress and total leaf chlorophyll (Fig. 1). The reduction in total chlorophyll was 40%, compared to the control plants (Fig. 1). Changes in the amount of carotenoids did not significant ($P > 0.05$). Essential oil increased more than 50% after 9 days without water. The soluble sugar and proline results will be described in the following section.

Metabolic profiling

Metabolite profiles of leaf extracts from three individual plants from each sampling time were compared (Table 2). Fifteen days after withholding water, soluble sugar accumulated 3.5 times more than the control plants. Three days after withholding water, the levels of the amino acid proline significantly increased, and after 15 days the levels were 15 times higher. Likewise, rosmarinic acid and caffeic acid also had significantly higher concentrations after water was withheld. The rosmarinic acid content increased up to 2.3 times

Fig. 1 Physiological parameters in response to water deficit in *S. rechingeri*. Effect of irrigation withhold on leaf water potential (a), relative water content (b), volumetric soil moisture (c), total Chl (d),

carotenoid (e), and oil yield (f) in 15 days. The bars represent $\pm$ SE. Bars labeled with different letter show significant differences ($P < 0.01$) by Duncan multiple range test

more than the control plants ($P < 0.01$) after 12 days of withholding water ($P < 0.01$), indicating that under severe stress conditions, rosmarinic acid production can be enhanced in the leaves of *Satureja* plants. The highest content of caffeic acid was found in the day nine samples, which showed up to a 4.5-fold change ($P < 0.01$), but ursolic acid and carnosic acid did not show significant changes. Carvacrol and thymol content increased 1.5 and 1.9-fold greater than that in the control plants after 9 days of withholding water ($P < 0.01$). p-Cymene increased up to 3.3-fold greater than that in the control plants after 15 day γ -Terpinene was not markedly changed among the different treatments.

Selection of appropriate reference genes for gene expression study

To assess the stability of the reference genes across different sampling times, the transcript abundances of the four candidate reference genes were detected by their mean Ct values, which ranged from 16.4 for *ACT* and *GAPC* to 23.9 for *UBC*. The mean of the Ct values of the three targeted genes was 22.7. Across all four reference genes, *ACT* was the most abundantly expressed, with the lowest average $Ct \pm SD$ (16.43 ± 0.7), followed by *GAPC* (16.49 ± 0.61), *UBC* (23.96 ± 0.59), and *ELF1- α* (18.54 ± 0.12 ; Table 1). The small coefficient of variation of the Ct value indicates that a given gene was more stably expressed. Among these

four candidate reference genes, *ACT* showed the greatest variation, with a coefficient of variance (CV) value of 4.2%, whereas *ELF1- α* showed the least expression level variation (0.45%) across all tested samples. The ranking of gene stability by CV was as follows: *ELF1- α* > *UBC* > *GAPC* > *ACT* (Table 1).

Stability analysis of reference genes by NormFinder, BestKeeper and geNorm

Three softwares were used to determine the stability of reference genes. The NormFinder determined the stability of reference genes based on inter- and intragroup expression variance. Analysis by NormFinder predicted that *UBC* and *GAPC* were the most stable in different drought sampling time (Table 3). The BestKeeper evaluated the stabilities based on the coefficient of variation (CV) and the standard deviation (SD) of the average Ct values. The genes with SD greater than one were excluded and considered unacceptable and those that showed the lowest CV and SD ($CV \pm SD$) were identified as the most stable genes (Xiao et al. 2014). Among reference genes *ELF1- α* had the lowest $CV \pm SD$ values (0.12 ± 0.45), indicating that *ELF1- α* 's expression was remarkably stable. These results were conflicting, with those acquired from the geNorm and NormFinder methods (Table 3).

Table 2 Metabolite profiling of *S. rechingeri* under drought stress (five sampling time after withholding water)

Metabolite	Sampling time (day)																				
	3				6				9				12				15				
	Average	SE	x fold change		Average	SE	x fold change		Average	SE	x fold change		Average	SE	x fold change		Average	SE	x fold change		LSD 1%
Soluble sugar ($\mu\text{g g}^{-1}$ FW)	1365.00	31.22	2.00		2125.15	27.95	3.12		2178.19	43.11	3.20		2146.12	98.67	3.15		2385.76	186.69	3.50		311.15
Proline ($\mu\text{g g}^{-1}$ DW)	0.56	0.56	2.35		0.94	0.13	3.95		1.48	0.10	6.24		3.58	0.25	15.10		3.56	0.29	15.01		0.87
Rosmarinic acid (mg g^{-1} DW)	24.15	24.15	0.98		25.71	0.58	1.04		48.96	1.18	1.99		56.97	0.90	2.31		37.00	0.35	1.50		2.71
Caffeic acid (mg g^{-1} DW)	3.90	3.90	1.31		4.52	0.21	1.52		8.20	0.32	2.75		13.50	0.08	4.53		12.10	0.30	4.06		3.23
Ursolic acid (mg g^{-1} DW)	14.38	14.38	1.08		19.66	1.20	1.48		17.38	2.11	1.31		13.44	0.79	1.01		12.40	0.61	0.93		4.32
Carnosic acid (mg g^{-1} DW)	3.54	3.54	0.93		5.23	0.37	1.38		4.36	0.16	1.15		4.23	0.16	1.11		3.48	0.18	0.92		0.89
Carvacrole (mg g^{-1} DW)	42.74	42.74	1.23		45.87	2.17	1.32		54.57	3.47	1.56		53.71	2.56	1.54		52.48	1.83	1.50		1.04
Thymol (mg g^{-1} DW)	0.12	0.12	1.49		0.18	0.01	2.12		0.16	0.01	1.96		0.16	0.01	1.93		0.13	0.00	1.52		0.09
γ -Terpinene (mg g^{-1} DW)	0.59	0.59	0.84		0.94	0.14	1.34		0.81	0.11	1.14		0.59	0.16	0.84		0.76	0.02	1.08		0.84
p-Cymene (mg g^{-1} DW)	0.08	0.08	1.90		0.11	0.04	2.60		0.08	0.04	1.92		0.13	0.03	2.98		0.15	0.03	3.34		0.28

The fold change relative to control is indicated in a separate column in italics. This value was calculated from the average values of the metabolites in 3, 6, 9, 12, and 15 days after withholding water

Table 3 Expression stability of candidate reference genes as calculated by Normfinder, Bestkeeper, geNorm, and comprehensive ranking in *S. rechingeri*

Normfinder		Bestkeeper			geNorm		Comprehensive ranking	
Gene	Stability	Gene	SD	CV	Gene	M value	Gene	Stability
UBC	0.372	ELF1a	0.12	0.45	UBC	0.619	UBC	1.19
GAPC	0.541	UBC	0.59	2.47	ACT	0.619	ACT	2.45
ACT	0.580	GAPC	0.61	3.69	GAPC	0.711	GAPC	2.45
ELF1a	0.625	ACT	0.70	4.28	ELF1a	0.756	ELF1a	2.83

The geNorm determined the stabilities based on *M* value and candidate the optimum reference gene (s) in different sampling times of drought treatment and species. Since a lower *M* value indicates increased stability, four genes were arranged according to their *M* values. As shown in Table 3, *UBC* and *ACT* were determined to be the most stable reference genes. In contrast, *GAPC* and *ELF1a* were the least stable reference genes.

Reference gene validation

To obtain a consensus result of the most stable reference genes as recommended by the three methods according to the RefFinder approach (Xie et al. 2012; <http://150.216.56.64/referencegene.php?type=reference>), the geometric mean of three algorithms corresponding rankings for each candidate gene was calculated (Table 3). *UBC* was the most stable reference genes. To validate the selected reference gene for normalization, the expressions of *HMGR*, *DXR*, and *RAS* were analyzed using RT-qPCR under different sampling times after water was withheld. The results showed that the expression levels of the three targeted genes and the selected reference gene had similar trends, but the quantity of the targeted genes was more when *UBC* was used as a reference gene. These results clearly indicate that using an improper reference gene could lead to misleading results for the normalization of the target gene. Thus, the results of the analysis further highlight the importance of selecting an appropriately stable reference gene prior to RT-qPCR study to avoid low accuracy.

Quantitative real-time RT-PCR analysis (RT-qPCR)

To determine whether drought stress affects the expression levels of *DXR*, *HMGR*, and *RAS* genes, transcript quantification of these genes was performed via RT-qPCR analysis to find the transcript level changes of these genes at sampling times of 3, 6, 9, 12, and 15 days after discontinuing irrigation. The expression level was normalized against the normal transcript level of *UBC*, which were taken as the reference genes for this experiment. Melt curve analysis was determined at only one peak point for each gene. Quantitative analyses of three targeted genes (*DXR*, *HMGR* and *RAS*) at

different sampling times were done by REST 2009 software, and the analysis of variance showed significant up-regulation in the expression of all three genes. The corresponding analyses revealed that the presence of drought stress drastically enhanced the relative expression of *DXR* and *RAS* 9 days after discontinuing irrigation (Fig. 2). The relative expression of *DXR* and *RAS* increased up to 18-fold and 41-fold 12 nine days after discontinuing irrigation (Fig. 2). The continuation of drought stress leads to decreased three genes expression. The trend of *HMGR*-relative expression was similar to *DXR* and *RAS*; it increased up to 10-fold until the ninth day, but the continuation of stress caused decreased *HMGR*-relative expression.

Discussion

The purpose of this study was to investigate whether increases in the biosynthesis of natural compounds (including terpenoids) result from increases in the relative gene expression of secondary metabolites. Another objective was to obtain more information about the effects of drought stress on the accumulation of monoterpenes and evaluate secondary metabolism in savory species. GC/MS and HPTLC analyses showed that the maximum amount of essential oil compounds related to carvacrol and thymol belonged to two cyclic monoterpenes. Rosmarinic and caffeic acids were the most important compounds in the extract and were significantly affected by drought stress.

Effects of drought stress on physiological parameters

RWC and LWP are effective tools to rapidly estimate physiological indices which are directly related to soil water content. Among the screening characteristics tested in the current study, survivability was found to be a valid and reliable tool for screening drought-tolerant plants. This study's experiments identified *S. rechingeri* as drought tolerant after 15 days without irrigation. In another study differences in drought adaptation among genotypes of *Thymus* species were found and can be attributed to differences in morphological and physiological responses to

Fig. 2 Expression profile of *DXR*, *HMGR*, and *RAS* genes (*UBC* as the reference gene) in *S. rechingeri* under drought stress. Data are expressed as the mean $\pm$ SE of three independent biological replicates and three technical replications. Bars labeled with different letter show significant differences ($P < 0.01$) by Duncan multiple range test

water availability (Moradi et al. 2017). Osmotic adjustment in drought-tolerant plants might be an active accumulation of solutes (such as soluble sugar, proline, etc.) in response to decreases in soil water potential (Rivas-ubach et al. 2014), thus enabling the plant to maintain turgor (lower cellular osmotic potential) and survive longer. In some conditions, RWC is not an adequate measure of water status, and LWP should be applied. LWP provides a more accurate index of available water in tissues with high lipid levels (which have lower water content) or if sampling is done in non-equilibrated (heterogeneous) tissues (Wu et al. 2016).

Volumetric soil moisture in *S. rechingeri* decreased as soil water deficit decreased. This outcome agrees with results reported on *Quercus ilex* trees by Rivas-ubach et al. (2014). Reductions in the value of total chlorophyll but not carotenoid were detected. At the end of the drought stress period, the uppermost leaves started wilting and turning yellow. Reduction of chlorophyll content in response to drought stress has been shown in previous studies such as Ekren et al. (2012) in *Ocimum basilicum* L. and Rivas-ubach et al. (2014) in *Quercus ilex* trees. In the present study, essential oil increased significantly ($P < 0.01$). Padacsi (2014) reported that the highest value of essential oil concentration in summer savory (*Satureja hortensis*) was measured as 50% of the soil water capacity. Ekren et al. (2012) showed that the essential oil ratio of *Ocimum basilicum* L. increased as the applied amount of irrigation water decreased.

Effects of drought stress on metabolite profiling and gene expression

This study's results show that water deficit has an increasing effect on the accumulation of solutes such as proline, a major organic osmolyte, and soluble sugar. Moreover, since the proline and soluble sugar are multifunctional metabolites and protect plants from stress by contributing to cellular osmotic adjustment, reactive oxygen species (ROS), detoxification, and protection of membrane integrity, increases in these solutes in *S. rechingeri* indicate this species' continued adaptation to drought stress. Proline's role in protecting plants against drought stress is well documented. Khan et al. (2016) have provided a list of transgenic plants engineered with genes catalyzing the biosynthetic pathways of proline that enable the plant to counteract osmotic stress. Proline plays various roles during stress such as metal chelator, anti-oxidative defense molecule, and signaling molecule (Zhang et al. 2014). Another important part of the non-enzymatic defense system is the accumulation of low molecular weight, water-soluble sugars as osmolytes. Since the sugar content was increased 3.5-fold, it can be assumed that sugar accumulation plays a more significant role in drought-tolerant species. Osmotic potential depends on the number of molecules in the material; thus, osmotic regulation can be achieved by converting polysaccharides (starch, fructan) to each other and joining oligosaccharides (sucrose, glucose) together (Hendry and Wallace 1993). The physiological action of

these sugars inhibits the connection between the neighboring membranes during water deficit and leads to lipid storage and protein stability (Ho et al. 2001).

In the current study, caffeic, rosmarinic, ursolic, and carnolic acids were measured using HPTLC and showed considerable increases for caffeic and rosmarinic acids in response to drought stress. The pattern of change revealed a significant increase ($P < 0.01$) in the amount of metabolites with the onset of stress, but with continuing stress, a rapid drop was detected. These results show a trend similar to other reported studies. Sabra et al. (2012) reported that the effects of salinity-induced changes in alkaloids, ketones, and caffeic acid derivatives (cichoric acid, caftaric acid, chlorogenic acid, and cynarin) in three *Echinacea* species. Notable changes in their relative amounts were found, depending on species and salinity intensity.

Rivas-ubach et al. (2014) reported that moderate experimental drought increased the concentrations of sugars and polyphenolic compounds with antioxidant function in the leaves of *Quercus ilex* trees. Turtola (2005) reported that drought stress induced the concentrations of terpenes in conifers to increase, while in willows, the concentrations of phenolics decreased. High accumulation of phenolic compounds in aerial plant tissues was shown to be involved not only in adaptations to abiotic stresses such as salinity, drought, UV and heat radiation, chilling, and high light intensity but also in defense mechanisms against pathogens and herbivory (Sabra et al. 2012; Selmar and Kleinwächter 2013). Metabolites such as thymol, carvacrol, γ -Terpinene and p-Cymene were measured using GC/MS showed significant changes in response to drought stress. These results agree with studies by Selmar and Kleinwächter (2013) and Radwan (2014) in which drought stress increased the concentration of monoterpenes in sage plants and Chapman (2009), where the application of water deficit significantly induced terpene concentration for multiple mono- and sesquiterpenes (e.g., α -pinene, β -phellandrene, α -cubebene, and caryophyllene) in lemon verbena (*Aloysia citriodora*) and markedly reduced the foliar biomass yield per plant. Padacsi (2014) reported that different water supply levels influenced the essential oil composition of basil; the ratio of linalool decreased (10–15%), while 1,8-cineole and tau-cadinole increased when water was withheld.

Reference gene validation

A suitable reference gene should be stably expressed across all samples and under different experimental conditions. Therefore, it is important to find a suitable reference gene appropriate for RT-qPCR normalization in the tissue or experimental condition of interest (Wu et al. 2016). To the authors' knowledge, the current study is being the first to systematically select appropriate reference genes for RT-qPCR

analysis in savory. Previously, *ACT* had been considered the most stably expressed gene, and it has been widely used as the reference gene for expression normalization in many plants (Ma et al. 2016). In the present study, *UBC* showed one of the most stably expressed reference genes as determined by geNorm and NormFinder. It was identified as one of the top-ranked reference genes in this study. One of the most important criteria for selecting a reference gene is the approximation of its Ct to the Ct of targeted genes. *UBC* was stably expressed under different drought stress treatments. In the current study, *ACT* generally ranked as second, third and fourth when analyzed using the three algorithms. Despite this gene's stability under different drought treatments, its Ct differed from the targeted genes, indicating that it may be unsuitable as a reference gene for gene expression normalization. The top-ranked gene identified by the three different algorithms was *UBC* and the comprehensive comparison verified that this gene is suitable. A prior study on *Salvia miltiorrhiza* (Lamiaceae family) showed that *ACT* and *UBC* were the most stable genes, whereas *ELF1- α* did not favor the normalization of RT-qPCR results (Yang et al. 2010). This study's findings provide important guidelines for selecting appropriate reference genes under experimental drought conditions in *Satureja* species.

Relative expression of three genes

To gain a new perspective on the mechanisms of drought tolerance in savory species, the relative expression of genes coding for enzymes involved in the biosynthesis of important metabolites were examined using biochemical and molecular approaches. In the present study, two key genes from terpenoids biosynthetic pathways (*MVA*, *MEP*) and one gene from the phenylpropanoid biosynthesis pathway were examined. These upstream genes were chosen, because the downstream genes involved in secondary metabolite pathways biosynthesis were not isolated. The relative expression levels of *DXR*, *HMGR*, and *RAS* genes in response to drought stress indicate that their relative expression may increase the biosynthetic capacity of monoterpenes. *RAS* levels increased soon after the beginning of stress, indicating the species' rapid response as well as its ability to stimulate the biosynthesis of rosmarinic acid and caffeic acid. In the present study, the level of substances within the essential oil varied after discontinuing irrigation (different sampling), leading to increased substances compared with the control plants. The results show that the expression of terpenoid and phenolic metabolism-related genes responded differently to drought stress. In drought-stressed leaves, *DXR*, *HMGR*, and *RAS* genes were upregulated to a much greater extent than those at the first sampling time. The relative expression of *DXR* and *HMGR* genes was also positively related to the amount of thymol. Therefore, raising the biosynthesis

of terpenoids represents an additional tool in stress conditions that the excess energy of cells is used appropriately. Thus, to maintain balance in reactions, as the $\text{NADPH} + \text{H}^+$ content increases, monoterpenes synthesis also increases (Selmar and Kleinwächter 2013). In *Cistus creticus*, the expression profiles of genes coding for enzymes from the MVA and MEP pathways were examined in different tissues in response to abiotic stresses. The results showed that the strongest alterations in the transcript levels related to *DXS* and *HMGR* genes (the rate-limiting steps of the isoprenoids' pathways), which correspond to the regulatory steps of the MVA and MEP pathways (Pateraki and Kanellis 2010).

In this study, *DXR*, as one of the corresponding biosynthesis genes, had approximately 18-fold higher transcript levels than control 12 days after discontinuing irrigation. Thymol and carvacrole, two of the metabolites detected, might be affected by increases in the relative gene expression of *DXR*, as it was significantly increased. Nevertheless, *DXR* and *HMGR* produced upstream substrates for carvacrol and thymol, which showed significant increases (Table 2). In the present study, free proline contents accumulated in the savory leaves 3 days after water was withheld (twofold), suggesting that the delayed response of *Satureja DXR*, *HMGR*, and *RAS* genes to dehydration, may lead to the efficient protection of photosynthetic apparatus in the leaves.

Conclusion

The results presented in current study show the effects of drought stress on secondary metabolism in savory species. Next, the important compounds such as carvacrol, thymol, and rosmarinic acid and caffeic acid should be increased using metabolic engineering in drought-tolerant species of savory. The authors established that the investigated physiological parameters (RWC, water potential, Chl content) effectively represent the ratio of drought stress. The quantity and the quality of *Satureja* compounds including essential oils and ethanolic extract changed. Drought stress also significantly affected key genes.

1. The authors determined that differences in RWC and LWP might play an important role in the drought tolerance of *S.rechingeri*. LWP decreased intensively in response to lower volumetric soil water.
2. Chlorophyll, soluble sugar, proline content, and essential oil density appear to be suitable features to characterize the effects of drought stress. Note that the maximum increase in essential oil was 50% (after 9 days of withholding water).
3. The authors present a novel systematic attempt to validate a set of candidate reference genes for the normalization of gene expression using RT-qPCR in savory under

drought stress conditions. The expression stability of the four candidate genes was analyzed by three applications (geNorm, NormFinder, and BestKeeper), and their results were further integrated into a comprehensive ranking. To study gene expression under drought stress, *UBC* is recommended to normalize the RT-qPCR data. This study also highlights the importance of identifying suitable reference genes for different plant species and various experimental conditions.

4. As the result of significant changes in several metabolites' production and the quantity of essential oil yield, a notable increase in the transcript levels of three targeted genes that affected the production of terpenoids and phenolic compounds can be expected. As the reference genes *UBC* met expectations, they should be used for future work in savory species.

In conclusion, the combination of metabolite profiling and physiological parameters contributed to greater understanding of the mechanisms of the *Satureja* plant's response at the metabolomics level. Moreover, the results suggest that the quality of the raw material obtained from *S. rechingeri* exposed to drought stress (12 days after withholding water) would be higher than that obtained from non-stressed plants. This species is a promising candidate for metabolic engineering. What remains to be established is to understand the mechanisms involved and identify the sequences that confer this improved performance.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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