Drought Stress in Iranian Endemic Savory (*Satureja rechingeri*): *In vivo* and *In vitro* Studies

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Abstract

The importance of different species of savory is due to having phenolic compounds such as carvacrol and thymol in the essential oil and rosmarinic acid in the extract, having strong antioxidant and antimicrobial effects. This study was conducted on five ecotypes of *Satureja rechingeri* in *in vitro* and *in vivo* experiments. After determining the best shooting and rooting medium, micropropagation was done and clones were prepared. Water stress treatments were prepared by increasing agar up to 100% followed by selecting the most tolerant and sensitive clones. In the *in vivo* experiment, effect of water stress was studied in a greenhouse with irrigation withhold and sampling at five stages. Ten morphological and physiological characters were recorded from both experiments. The tolerant clone had superior tolerance to water deficit. Increasing the agar concentration up to 66% had no effects on rooting and the growth rates of shoots but more increase led to a sharp reduction in the growth rate and root differentiation. In the greenhouse, the tolerant clone was tolerated the stress up to nine days without showing any symptoms, but the continuation of stress led to a sharp increase in proline and soluble sugars and the reduction of plant pigments and leaf osmotic potential. This research was a kind of drought simulation at *in vitro* condition, performed for the first time for identification and screening of drought tolerant clones of savory.

Keywords: Drought stress; Micropropagation; Morphological and physiological characteristics

Introduction

Savory (*Satureja spp*.) is one of the most important medicinal plants in Iran, belonging to the family of Laminaceae and about 284 species have been identified worldwide. Iran as one of the world's most important sources of germplasm has 16 species of the genus *Satureja* (Hadian *et al.* 2014). Different species of savory due to its analgesic, disinfectant, antioxidant, antiviral, anti-microbial, antifungal, anti-inflammatory and anticancer properties are used in the treatment of many diseases (Momtaz and Abdollahi 2010). The main constituents of the essential oil of *Satureja rechingeri* are thymol and carvacrol (Sefidkon *et al.* 2004) and this species is mainly distributed in Ilam province, West of Iran (Hadian *et al*. 2014).

Drought is one of the environmental stresses which is considered as a major barrier to agricultural production in the world. For screening of drought tolerant genotypes in laboratory, polyethylene glycol (PEG), mannitol or sorbitol have been used so far (Ruf *et al.* 1967; Corchete and Guerra 1986; Wani *et al*. 2010; Roy *et al.* 2011; Ullah *et al.* 2014), but the toxicity of PEG for living organisms has caused the reduction of plant's growth. Impurities such as ethylene oxide and 1,4-dioxane are the cause of toxicity of this substance (Andersen 1999). This causes error in the results so that the researcher cannot separate toxic effects from the effects of decrease in osmotic potential. *In vitro* screening is based on induction of the genetic diversity among either regenerated populations, cells, tissues or organs of

a plant (Rai *et al.* 2011). In this method, the period of screening is also reduced due to the minimized environmental impacts (Jain 2001). Despite the many benefits of *in vitro* screening, there are restrictions such as reduced regeneration during the screening as well as the phenomenon of epigenetic adaptation (Tal 1994). Sometimes, non-tolerant cells are exposed to epigenetic adaptation which is heritable only through mitotic division. There is some evidence indicating epigenetic changes that is caused by DNA methylation in tissue culture experiments (Guo *et al.* 2007; Gao *et al.* 2010). In the *in vitro* experiments many epigenetic variations could be prevented by shortening the screening period and or reducing the stages up to one (Tal 1994). High cost and facilities required for cultivation and the care of genotypes are among the problems for screening the resistant genotypes in the field, of which the most critical part is how to properly apply the stress. Due to the variability of many factors including non-homogeneous soil, difference in irrigation method, poor drainage, climate change, pests and diseases and many other factors, the accuracy of the investigation is reduced. This research was aimed to examine the relationship between the response to drought stress under *in vitro* and greenhouse conditions and development of an easy screening method in order to generate tolerant clones of savory before large-scale planting.

Materials and Methods

Preparation of plant material and micropropagation to produce clones

The studied ecotypes were collected from five locations of Iran [four ecotypes from different locations of Ilam province (E1, E3, E4, E5) and the other one from Lorestan (E2)]. Healthy branches were placed under running water for 2 h, soaked in 2% (v/v) Vitavax for 20 min and sterilized with 70% (v/v) ethanol and 1.5% (v/v) sodium hypochlorite for 30 s and 3 min, respectively. Then, they were chopped into the pieces of 1-1.2 cm length and were cultured in the MS culture medium containing IBA (0.01 mg l^{-1}), BA (0.3 mg l^{-1}) and 2ip (0.3 mg l^{-1}). Five weeks after the establishment of explants, one of the ecotypes (E3), established in large numbers, was selected to be used in the micropropagation experiments in order to determine the best hormonal composition for shooting and rooting. Ten different culture media with various compositions of growth regulators were prepared to propagate the established seedlings (Table 1). After five weeks, in order to select the best hormonal treatment, the traits such as number of main branches, number of active lateral buds, length of main branch, leaf color and number of leaves were recorded. Leaf color density visually scored by 1-5 scale to evaluate the leaf color, five and one being the maximum and the minimum green color, respectively. After shoot proliferation, eight rooting treatments were prepared (Table 1) to determine the best hormonal composition for rooting, and after a certain period of time (5 weeks), the traits such as root length, number of primary and secondary roots as well as shooting traits were assessed. The pH of the culture medium was adjusted to 5.6 and the plants were grown in a growth chamber with a photoperiod of 16 h light and a temperature of 25 ± 0.1 °C.

Optimization of stress treatments at *in vitro* conditions

This preliminary experiment was performed to optimize the type and the level of water stress and it was used as a factor to apply stress in the next step. Eight stress treatments including 6, 8, 10, 12 and 14 g l^{-1} agar and two treatments of 5 and 10 wt% PEG 6000 were applied. These amounts were added to the MS culture medium containing IBA (0.1 mg l^{-1}) as the best concentration of hormonal treatment for rooting. After about a month, shoot length, root length and seedling fresh weight were measured. Results showed that the use of PEG led to the loss and yellow color of new branches. A further increase of more than 12 g l^{-1} agar also led to the excessive rigidity of culture medium as well as the growth inhibition of explants. As a result, appropriate treatments of concentrations of 6, 8, 10 and 12 g agar were just selected.

Preparation and applying stress treatments at in *vitro* conditions

In order to prepare the drought treatments, MS medium containing the best hormonal composition for rooting (0.1 mg l^{-1} IBA) was prepared and different doses of agar including control (6 g l^{-1}), 8, 10 and 12 g l^{-1} were added so that the agar content increased 33%, 66% and 100% in different treatments compared to the control. After about a month, 10 different traits including shooting, rooting and fresh and dry weight of seedlings were evaluated and the tolerant and the sensitive clones were selected and were transferred to the greenhouse.

Drought stress in the greenhouse

After the adaptation and establishment in the greenhouse, two clones were transferred to the pots of 30 kg and irrigated regularly for three months. Then, the morphological traits including plant height, number of primary and secondary branches and largest and smallest diameter of canopy were measured. In order to compare drought tolerance of two clones, irrigation was discontinued for two weeks and 10 characters were measured every three days. The traits were leaf water potential (MPa), relative water content (RWC; %), chlorophylls (mg g^{-1} FW; total, a, b, a/b) (Chl), carotenoid (mg g^{-1} FW) (CAR), soluble sugar (μg g^{-1} FW), proline (μg g^{-1} DW) and essential oil yield (%). The volumetric soil water content was also measured by TDR. Leaf water potential was measured using the liquid immersion method (Michel 1972). RWC (%) was calculated using the following equation:

$$RWC = \frac{(FM - DM)}{(TM - DM)} * 100$$

In the above equation, FM, DM and TM stand for fresh mass, dry mass and turgid mass, respectively (Boyer 1968). Total soluble sugars were measured using the anthrone method (Irigoyen *et al.* 1992). Proline content was measured based on fresh weight according to the method of Bates *et al.* (1973). Plant pigments were extracted with acetone method (Lichtenthaler and Welburn 1983). Essential oils were extracted by water distillation method (Clevenger) for three hours. The yield of essential oil was calculated from the percentage of essential oils obtained from 100 g of dry matter.

Table 1. Different hormonal treatments used for shooting and rooting experiments

Shooting treatments	Oxin (mg l⁻¹)					Cytokinin (mg l⁻¹)				Rooting treatments	Oxin (mg l⁻¹)				
	IBA		BAP			TDZ	Kin	2ip			IBA			NAA	
	0.01	0.1	0.1	0.3	0.5	0.05	0.2	0.3	0.5		0.1	0.5	1	0.1	0.5
S1	+	-	-	-	+	-	+	-	-	R1	+	-	-	-	-
S2	+	-	-	+	-	-	+	-	-	R2	-	+	-	-	-
S3	+	-	+	-	-	-	+	-	-	R3	-	-	-	+	-
S4	-	+	-	+	-	-	-	+	-	R4	-	-	-	-	+
S5	+	-	-	-	+	-	-	-	+	R5	+	-	-	-	+
S6	+	-	-	+	-	-	-	-	+	R6	-	+	-	-	+
S7	+	-	-	-	+	-	-	+	-	R7	-	+	-	+	-
S8	+	-	-	+	-	-	-	+	-	R8	-	-	+	-	-
S9	+	-	-	-	-	+	-	+	-						
S10	+	-	-	+	-	+	-	-	-						

+ , - : The presence and the absence of the hormone, respectively.

Statistical analysis

Prior to analysis of variance (ANOVA), test for the homogeneity of variances was performed and the normality of data was tested by SPSS16.0 statistical software package. Variables with normal distribution were analyzed by one-way ANOVA ($P \leq 0.05$) and Duncan's Multiple Range Test, and in cases where the data were not normally distributed, the data transformation methods were used. On the other hand, in cases where the data were not on the interval scale, Kruskal-Wallis test was used for group comparisons (McDonald 2008). ANOVA of the effect of drought stress on two clones in the greenhouse conditions was conducted in a factorial experiment on the basis of a completely randomized design (CRD) with two factors, including clone (two levels) and sampling time (six levels). The mean comparison of the effects of drought stress on the two clones was performed by *t*-test.

Results and Discussion

Results of the effects of different shooting treatments on number of main branches, number of active lateral buds, length of main branch, leaf color and number of leaves are presented in Table 2a. Different hormonal treatments had significant effect on all measured traits. The highest number of main branches, active lateral buds, leaf color and number of leaves were obtained from S6, containing IBA (0.01 mg l⁻¹), BA (0.3 mg l⁻¹) and 2ip (0.5 mg l⁻¹; Table 3a). Figure 1a also shows the superiority of S6 compared to other treatments. In this figure, both S9 and S10 treatments had negative effect on shooting. Table 2b indicates that the rooting treatments had significant effects on the root length, number of primary and secondary roots and branch length. However, no significant effects were recorded for number of primary branches, number of active buds and leaf color. The rank of each rooting treatment for the studied traits is shown in Table 3b. The first rooting treatment containing IBA (0.1 mg l⁻¹) resulted in the longest root and the highest number of primary roots. However, R2, R3 and R8 containing IBA (0.05 mg l⁻¹), IBA (1 mg l⁻¹) and NAA (0.1 mg l⁻¹) were also suitable treatments but R5, R6 and R8 containing a mixture of IBA and NAA resulted in the minimum rooting (Figure 1b).

Table 2. Results of hormonal shooting a) and rooting treatments b) on the measured characters by Kruskal Wallis Test; c) propagated *Satureja* clones were evaluated by *in vitro* drought stress treatments

	Statistical analysis	Root length (mm)	Main roots (No.)	Lateral roots (No.)	Shoots (No.)	Active buds (No.)	Shoot length (mm)	Leaf color	Leaves (No.)	Fresh weight (mg)	Dry weight (mg)
a	χ^2				22.36^{**}	39.2^{**}	42.18^{**}	46.63^{**}	44.8^{**}		
	Df				9	9	9	9	9		
b	χ^2	35.3^{**}	23.1^{**}	34.7^{**}	9.6^{ns}	5.8^{ns}	15.9^{*}	13.8^{ns}			
	Df	7	7	7	7	7	7	7			
c	X^2	16.56^{**}	17.78^{**}	13.54^{**}	4.45^{ns}	16.13^{**}	4.53^{ns}	7.80^{ns}	7.22^{ns}	12.15^{**}	13.60^{**}
	Df	4	4	4	4	4	4	4	4	4	4

ns, *, ** Nonsignificant (*P*>0.05) and significant at *P*≤0.05 and *P*≤0.01, respectively.

Figure 1. Effects of ten shooting (a) and eight rooting treatments (b) on *Satureja rechingri*. For treatments names refer to Table 1. Scale bars= 2 cm

Effects of drought stress on five clones at the *in vitro* condition

After determining the best shooting and rooting treatments, five clones were regenerated through micropropagation from five ecotypes and placed under drought stress at the *in vitro* condition. ANOVA of the effects of drought treatments on root length, the number of primary and secondary roots, number of active lateral buds, fresh and dry weights of seedlings were statistically significant but no significant effects were found for the number of primary branches, length of primary branch, leaf color and number of leaves (Table 2c). According to Table 3c, the maximum growth in terms of root length, number of primary and secondary roots, number of primary branches,

number of active lateral buds, number of leaves, fresh and dry weights were recorded for clone C1 under drought stress at the *in vitro* condition. Clone C2 showed minimum growth for aforementioned traits. According to the results of this stage, clones C1 and C2 were selected as the most tolerant and the most sensitive clones, respectively and transferred to the greenhouse. The change in the trend of sensitive and tolerant clones after irrigation withhold is shown in Figures 2a and 2b. It should be noted that despite the normality of fresh and dry weights data and the possibility of analyzing these data by GLM and Duncan's Multiple Range Test, they were analyzed together with other traits by Kruskal-Wallis method to reduce the number of tables.

Table 3. Kruskal Wallis Test for ranking of a) 10 hormonal shooting treatments, b) eight rooting treatments and c) five propagated *Satureja* clones under *in vitro* drought stress for the measured characters. S and R definition are available in Table 1

	Treatment	Dry weight (mg)	Fresh weight (mg)	Leaves (No.)	Leaf color	Shoot length (mm)	Active buds (No.)	Shoots (No.)	Lateral roots (No.)	Main roots (No.)	Root length (mm)
a	S1			21.2^{c+}	4.4^b	6.3^b	1.8^e	1.2^b			
	S2			20.4^d	4.3^c	6.2^c	1.8^e	1.0^c			
	S3			18.8^e	3.5^e	5.7^d	1.2^g	1.0^c			
	S4			22.0^b	4.0^d	5.4^e	2.0^d	1.0^c			
	S5			22.4^b	4.7^a	5.7^d	6.0^b	1.0^c			
	S6			24.8^a	4.7^a	6.5^a	7.2^a	1.0^c			
	S7			21.2^c	3.0^f	3.9^f	1.6^f	1.0^c			
	S8			17.2^f	2.7^g	3.6^g	1.2^g	1.0^c			
	S9			12.8^g	2.4^h	2.9^h	2.8^c	1.0^c			
	S10			10.4^h	2.0^i	2.5^i	2.0^d	1.6^a			
b	R1				4.0^b	8.1^a	4.4^b	1.8^b	14.2^b	2.6^a	10.8^a
	R2				3.1^f	7.3^b	4.8^a	2.0^a	13.0^d	1.4^d	7.2^c
	R3				3.4^e	7.5^b	4.2^c	1.4^c	14.0^c	1.4^d	10.0^b
	R4				3.7^c	6.4^c	3.2^d	1.2^d	2.6^f	1.2^e	2.2^e
	R5				2.8^g	4.7^e	2.8^e	1.0^e	5.2^e	2.4^b	2.1^e
	R6				4.2^a	5.0^d	2.6^f	1.0^e	2.6^f	1.8^c	1.1^f
	R7				3.5^d	6.5^c	2.8^e	1.0^e	0.0^g	0.0^f	0.0^g
	R8				4.3^a	5.0^d	1.6^g	1.0^e	21.6^a	1.2^e	6.7^d
c	C1	0.12^d	0.90^e	22.9^a	3.8^d	5.2^e	5.6^b	1.2^a	15.2^a	2.1^a	6.5^a
	C2	0.13^c	1.01^c	19.4^c	3.5^e	5.6^c	3.2^d	1.1^c	5.0^e	0.4^e	1.8^d
	C3	0.12^d	0.94^d	18.9^e	4.2^b	5.7^b	3.2^d	1.1^c	6.3^c	0.9^b	3.7^b
	C4	0.15^b	1.14^b	19.5^b	4.3^a	5.5^d	6.5^a	1.1^b	5.6^d	0.5^d	2.6^c
	C5	0.15^a	1.17^a	19.1^d	4.1^c	5.8^a	4.6^c	1.0^d	6.8^b	0.7^c	2.7^c

+In each column and for each factor the values with different letter are significantly different at 0.05 probability level
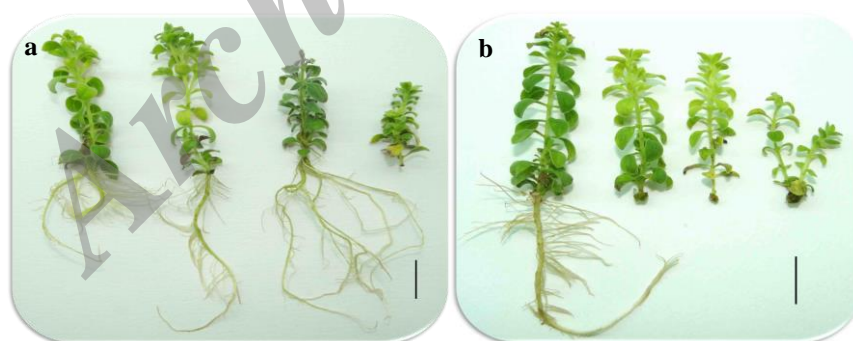

Figure 2. Comparison of different agar concentration in a) clone 1 and b) clone 2 of *Satureja rechingeri*. Scale bars = 2 cm

After transferring the two selected tolerant and sensitive clones to the greenhouse and their adaptation after three months, morphological characteristics including plant height, number of primary and secondary branches and the largest and the smallest canopy diameter were measured. Results showed no significant differences between the two clones for the mentioned traits. In the next step, irrigation was stopped for 15 days and sampling was conducted every three days and the

characteristics given in Table 4 were measured. ANOVA of the effects of the drought stress showed significant differences between the two clones for all assessed traits (except for soil moisture content). In terms of sampling time, significant differences were observed for all traits except the ratio Tof Chl a/b. Thus, significant differences were occurred in the magnitude of the measured traits over time.

Table 4. Effects of two clones of *Satureja rechingeri* during 15 days in greenhouse on the studied characters. a) ANOVA, b) comparison of C1 and C2. Reported values are mean ± S.E., P-values based on *t*-test for independent samples (n = 18)

	Source of variation	Oil yield (%)	Proline (µg g^{-1} FW)	Soluble sugar (µg g^{-1} DW)	CAR (mg l^{-1} FW)	Chl a/b	Chl b (mg l^{-1} FW)	Chl a (mg l^{-1} FW)	Total Chl (mg l^{-1} FW)	RWC (%)	Leaf water potential (MPa)	Volumetric soil water content
a) ANOVA	Clone	13.50**	0.110**	1105389.3**	3.33**	17.51**	19.92**	12.37**	14.06**	9.34**	2.77**	0.44^{ns}
	Date	3.27**	0.320**	1471793.2**	5.08**	0.82^{ns}	1.27**	2.41**	3.16**	3.69**	5.41**	4.33**
	Clone* Date	0.24*	0.007*	97375.1*	0.35^{ns}	0.35^{ns}	0.42^{ns}	0.65^{ns}	0.06^{ns}	0.39*	0.56**	0.49**
	Error	0.09	0.002	34777.37	0.11	0.41	0.21	0.23	0.13	0.1	0.03	0.36
b) *t*-test	Clone 1	3.62 ± 0.18	1.99 ± 0.35	1750 ± 142	11.38 ± 0.30	1.78 ± 0.04	0.60 ± 0.02	1.06 ± 0.03	1.70 ± 0.05	81.70 ± 2.92	-3.70 ± 0.45	11.2 ± 1.7
	Clone 2	2.51 ± 0.07	1.31 ± 0.26	1400 ± 89	10.45 ± 0.42	2.15 ± 0.05	0.36 ± 0.03	0.78 ± 0.06	1.17 ± 0.09	62.99 ± 3.82	-2.66 ± 0.30	11.2± 1.8
	p-value	< 0.000	< 0.000	< 0.000	0.001	< 0.000	< 0.000	< 0.000	< 0.000	< 0.000	0.001	0.88

^{ns}, **, ** Nonsignificant (*P*>0.05) and significant at *P*≤0.05 and *P*≤0.01, respectively

The interaction of clone × sampling time was also significant for leaf water potential, proline and essential oil yield. This means that the responses of two clones were not identical at different sampling times. Also, volumetric soil water content decreased significantly at different sampling times, but as expected due to the homogeneity of greenhouse soil, no significant differences were observed between the volumetric soil moisture of the two clones (Figure 3a). Also, mean comparisons of two clones (Table 4) showed significant differences between the two clones (P<0.01) for all investigated traits. In the tolerant clone, after the stress period, RWC was about 29% higher than that in the sensitive clone (Figure 3b). Also, the amounts of total soluble sugars and proline were higher (40% and 25%, respectively; Figures 3d and 3e). Oil yield of the sensitive clone was 46% lower than that of the tolerant clone (Figure 3f). The pigments were also considerably higher in the tolerant clone which can be interpreted such that drought stress causes a rapid degradation of Chl content in the sensitive clone whereas the tolerant clone was not affected. However, in the tolerant clone, Chl content of the control plants was higher than that of the sensitive clone in the same condition. With regard to the role of proline and soluble sugars as osmoprotectants (osmotic regulators), it can be expected that leaf water potential is considerably lower in the tolerant clone and this amount was less than 39% as can be seen in Table 4 and Figure 3. Lower leaf water potential in the tolerant clone indicates high stability of the membrane and more capacity of osmoprotectants such as soluble sugars and proline.

The results of this study showed that the screened tolerant clone of Iranian endemic savory (*Satureja rechingeri*) against drought in the greenhouse had lower leaf osmotic potential and higher osmoprotectants, leading to higher membrane stability and consequently higher RWC

in the clone C1. It seems that proline accumulation is considered as a part of stress tolerance system in this clone because it has increased up to 10 times, compared to the threshold value. Soluble sugars also increased to three times in the tolerant clone compared to the control. During the drought period, plant species reduce osmotic potential of their cells through the accumulation of sugars and proline content and it facilitates the movement of water into their leaves. Proline acts as an osmotic regulator (osmoprotectant) and the stabilizer of proteins and membranes during stress (Sivritepe *et al.* 2008). Also, soluble sugars, as the main organic solvents, that are accumulated in the vacuoles and in the times of drought stress, lead to osmotic stabilization (Hoekstra *et al.* 2001). The physiological function of these soluble sugars is to inhibit the connection between membranes and stability of proteins through hydrogen bonds with linear sequence (Ho *et al.* 2001).

The reduction of Chl and pigments in the present work correspond with the findings of Brito *et al.* (2003) for the effect of drought stress on *Olea europaea* spp. at *in vitro*. Reduced Chl content may be due to the activation of chlorophyllase and consequently Chl degradation or it is because of the reduction in Chl synthesis and changes in the membrane structure of thylakoids. This reduction leads to low efficiency of photosynthesis in plants. Therefore, the plants which are able to maintain their Chl can have more photosynthesis. Carotenoids are the last

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pigments which are decomposed and destroyed. Species having more carotenoid content would have a more effective defense and show more tolerance to water deficit stress as compared to reactive oxygen species (Kim *et al.* 2012). In the present study, the lower loss of carotenoids in the clone E1 indicated its greater tolerance.

Increased essential oil content in the present work in response of *Satureja rechingeri* to drought stress is consistent with the results of other researchers, reported in *Satureja hortensis* (Baher *et al.* 2002), *Ocimium basilicum* (Khalid 2006) and parsley (Petropoulos *et al.* 2008). Actually, metabolic pathways, responsible for the accumulation of natural compounds, are closely associated with growth condition and stress situation. The majority of reported studies on drought stress indicate the increased amounts of secondary metabolites such as phenols, terpenes and nitrogenous compounds and also alkaloids, cyanogenic glucosides or glucosinolates (Tuteja and Singh Gil 2013). In our study, since plant biomass was reduced as a result of water stress, it could be concluded that increased oil yield might be possible if plant density per unit land area increased. Khalid *et al.* (2010) showed that drought stress had significant effect on either increasing the percentage of oil or the content of compounds such as methyl eugenol, limonene and isomenthone in *Pelargonium odoratissimum* (L.), although, due to higher crop yield, significant increase was observed in the oil yield of the control plants as compared to the stressed plants.
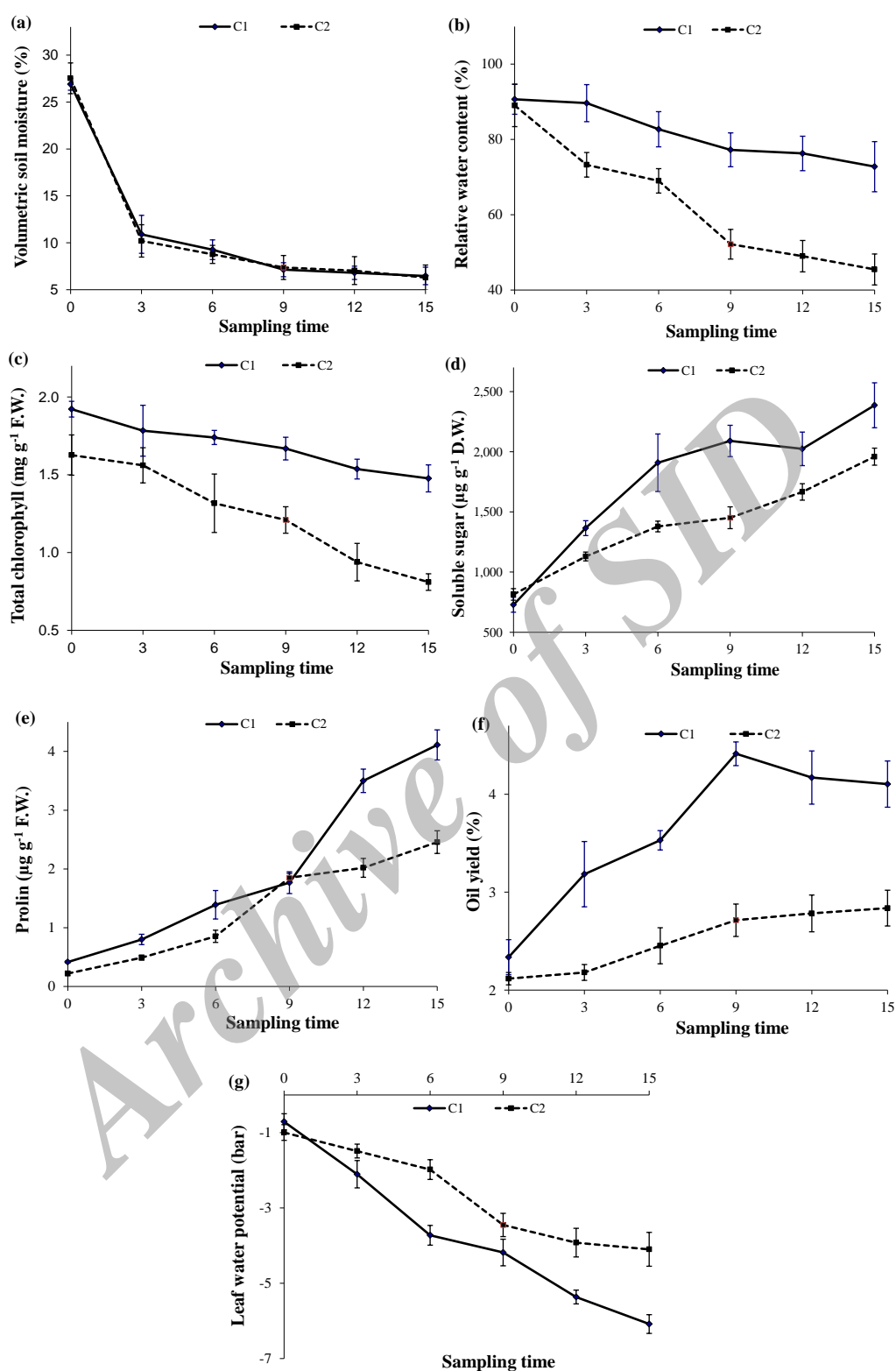
Figure 3. Difference between tolerant (C1, solid lines) and sensitive (C2, dotted lines) clones of *Satureja rechingeri* under drought stress. Effect of irrigation withhold on volumetric soil moisture (a), relative water content (b), total chlorophyll (c), soluble sugar (d), proline (e), oil yield (f) and leaf water potential (g) in 15 days. The bars show ± SE.

In the present study, reduced fresh and dry weight and decreased rooting capacity of five studied *Satureja rechingeri* clones were the consequences of drought stress in the *in vitro* condition. Roots were the first responsive trait to the drought stress for adapting the plant to drought and can serve as an important indicator to achieve drought tolerant genotypes. Considerable genetic diversity in the root activity and rapid production of lateral roots led to increased tolerance to drought in the clone E1. These results are in agreement with the *in vitro* studies carried out on figs and sweet cherry using PEG (Sivritepe *et al.* 2008; Karimi *et al.* 2012). Increased osmotic stress had adverse effect on the savory clones in both shoot and root proliferation stages in our study. Duncan *et al.* (1995), investigated the effects of drought stress at *in vitro* on *Sorghum bicolor* L. Moench and showed that although osmotic stress resulted in the reduction of regeneration potential, tolerant plants could be identified through the screening of regenerated plants and transferring them to the field. Many reports have been presented on applying drought stress at *in vitro* condition and the selection of susceptible (non-resistant) and resistant genotypes. Wani *et al.* (2010) presented a method for *in vitro* screening of drought- resistant transgenic rice using callus culture of two cultivars of rice in the medium containing different amounts of PEG 6000 and in this way resistant and non-resistant cultivars were identified. In another study, in order to assess the drought tolerance of tomato cultivars and observe the polypeptide changes during *in vitro* conditions, a number of factors including osmotic stress factors (PEG, mannitol and NaCl) and five levels of factors were examined. It was shown that to screen the plants, use of mannitol was better than PEG and NaCl for the selection of tolerant cultivar and expression of polypeptides (Roy *et al.* 2011). In another study, in order to evaluate the metabolic profile of *Lolium* in response to drought stress, after cloning from both non-resistant and resistant *Lolium*, hydroponic medium containing MS with 20% weight by volume (w/v) PEG 6000 was used as the drought stress treatment and then their metabolic profile were analyzed (Foito *et al.* 2009). In another report, the investigation of *in vitro* growth of banana varieties under osmotic stress conditions caused by sorbitol was used as a useful model for screening tolerant varieties. Also, tolerance homeostasis was identified using proteomics (Vanhove *et al.* 2012). In a study performed to select tolerant and non-tolerant species of *Triticum*, PEG 6000 was used in the hydroponic culture (Sultan *et al.* 2012). Unfortunately, the toxic effects of polyethylene glycol have been ignored in all previous works, mainly due to the inability to separate this effect from the effects of decreased water potential of the solution.

Conclusion

Among possible solutes that can influence osmotic adjustment, proline and soluble sugar accumulation differed in two *Satureja rechingeri* clones subjected to water stress. The clone which accumulated more soluble sugar and proline, had lower leaf water potential, higher RWC, Chl, CAR and oil yield. These results indicated that different ecotypes of *S. rechingeri* could have

different mechanisms in the solute accumulation that allows the plant to survive under drought conditions. In the present work, the micropropagation of savory (*Satureja rechingeri*) which is being reported for the first time, proved the positive relationship between the response to drought stress in *in vitro* and in *in vivo* (greenhouse) conditions. On the other hand, identifying factors involved in the drought tolerance as selection criteria and the easy screening method was presented to identify

tolerant clones of savory. What does now remain to be established is an understanding of the mechanisms involved and the identification of the sequences that confer this improved performance.

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References

Andersen FA, 1999. Special Report: Reproductive and developmental toxicity of ethylene glycol and its ethers. International Journal of Toxicology 18: 53-10.

Baher Z, Mirza M, Ghorbanli M and Rezaii MB, 2002. The influence of water stress on plant height, herbal and essential oil yield and composition in *Satureja hortensis* L. Flavour and Fragrance Journal 17: 275-277.

Bates IS, Waldern RP and Teare ID, 1973. Rapid determination of free proline for water stress studies. Plant and Soil 39: 205-207.

Boyer JS, 1968. Measurement of the water status of plants. Annual Review of Plant Physiology 9: 351-363.

Brito G, Costa A, Fonseca HM and Santos CV, 2003. Response of *Olea europaea* ssp. maderensis *in vitro* shoots exposed to osmotic stress. Scientia Horticulturae 97: 411-417.

Corchete P and Guerra H, 1986. Effect of NaCl and PEG on solute content and glycosidase activities during germination of lentil seeds. Plant Cell and Environment 9: 589-593.

Duncan RR, Waskom RM and Nabors MW, 1995. *In vitro* screening and field evaluation of tissue culture regenerated sorghum (*Sorghum bicolor* L. Moench) for soil stress tolerance. Euphytica 85: 373-380.

Foito A, Byrne SL, Shepherd T, Stewart D and Barth S, 2009. Transcriptional and metabolic profiles of *Lolium perenne* L. genotypes in response to a PEG-induced water stress. Plant Biotechnology Journal 7: 719-732.

Gao X, Yang D, Cao D, Ao M, Sui X, Wang Q, Kimatu JN and Wang L, 2010. *In vitro* micropropagation of *Freesia hybrida* and the assessment of genetic and epigenetic stability in regenerated plantlets. Plant Growth Regulation 29: 257-267.

Guo WL, Wu R, Zhang YF, Liu XM, Wang HY, Gong L, Zhang ZH and Liu B, 2007. Tissue culture induced locus specific alteration in DNA methylation and its correlation with genetic variation in *Codonopsis lanceolata* Benth et Hook F. Plant Cell Reports 26: 1297-1307.

Hadian J, Esmaeili H, Nadjafi F and Khadivi-Khub A, 2014. Essential oil characterization of *Satureja rechingeri* in Iran. Industrial Crops and Products 61: 403-409.

Ho S, Chao Y, Tong W and Yu S, 2001. Sugar coordinately and differentially regulates growth and stress related gene expression via a complex signal transduction network and multiple control mechanisms. Plant Physiology 46: 281-285.

Hoekstra FA, Golovina EA and Buitink J, 2001. Mechanisms of plant desiccation tolerance. Trends in Plant Science 6: 431-438.

Irigoyen JJ, Einerich DW and Sanchez Diaz M, 1992. Water stress induced changes in concentrations of proline and total soluble sugars in modulated alfalfa (*Medicago sativa*) plants. Physiologia Plantarum 84: 58-60.

Jain M, 2001. Tissue culture-derived variation in crop improvement. Euphytica 118: 153-166.

Karimi S, Hojati S, Eshghi S, Nazary Moghaddam R and Jandoust S, 2012. Magnetic exposure improves tolerance of Fig Sabz explants to drought stress induced *in vitro*. Scientia Horticulturae 137: 95-99.

Khalid KA, 2006. Influence of water stress on growth, essential oil and chemical composition of herb (*Ocimum basilicum* L.). International Agrophysics 20: 289-296.

Khalid KA, Teixeira da Silvab JA and Cai W, 2010. Water deficit and polyethylene glycol 6000 affects morphological and biochemical characters of *Pelargonium odoratissimum* L. Scientia Horticulturae 125: 159-166.

Kim SH, Ahn YO, Ahn MJ, Lee HS and Kwak SS, 2012. Down regulation of β-carotene hydroxylase increases β-carotene and total carotenoids enhancing salt stress tolerance in transgenic cultured cells of sweet potato. Phytochemistry 74: 69-78.

Lichtenthaler HK and Wellburn AR, 1983. Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. Biochemical Society Transactions 11: 591-592.

McDonald JH, 2008. Handbook of Biological Statistics. Sparky House Publishing, Baltimore, Maryland, pp. 153-160.

Michel BE, 1972. Solute potentials of sucrose solutions. Plant Physiology 50: 196-198.

Momtaz S and Abdollahi M, 2010. An update on pharmacology of *Satureja* species; from antioxidant, antimicrobial, antidiabetes and antihyperlipidemic to reproductive stimulation. International Journal of Pharmacology 6: 346-353.

Petropoulos SA, Daferera D, Polissiou MG and Passam HC, 2008. The effect of water deficit stress on the growth, yield and composition of essential oils of parsley. Scientia Horticulturae 115: 393-397.

Rai MK, Kalia RK, Singh R, Gangola MP and Dhawan AK, 2011. Developing stress tolerant plants through *in vitro* selection. An overview of the recent progress. Environmental and Experimental Botany 71: 89-98.

Roy R, Agrawal V and Gupta SC, 2011. Mannitol, polyethylene glycol and NaCl induced polypeptide changes during *in vitro* culture of three tomato cultivars. Biologia Plantarum 55: 591-595.

Ruf RH, Eckard ER and Gifford RO, 1967. Compound of osmotic adjustment of plants to rapid changes in root medium osmotic pressure. Soil Science 104: 159-162.

Sefidkon F, Jamzad Z and Mirza M, 2004. Chemical variation in the essential oil of *Satureja sahendica* from Iran. Food Chemistry 88: 325-328.

Sivritepe N, Sivritepe N, Erturk U, Yerlikaya C, Turkan I, Bor M and Ozdemir F, 2008. Response of the cherry rootstock to water stress induced *in vitro*. Biologia Plantarum 52: 573-576.

Sultan MARF, Hui L, Yang LJ and Xian ZH, 2012. Assessment of drought tolerance of some *Triticum* species through physiological indices. Czech Journal of Genetics and Plant Breeding 48: 178-184.

Tal M, 1994. *In vitro* selection for salt tolerance in crop plants: Theoretical and practical considerations. *In Vitro* Cellular and Developmental Biology-Plant 30: 175-180.

Tuteja N and Singh Gil S, 2013. Plant Acclimation to Environmental Stress. Springer, New York, USA, pp. 197-232.

Ullah I, Akhtar A, Mehmood N, Shah IA and Noo M, 2014. Effect of mannitol induced drought stress on seedling traits and protein profile of two wheat cultivars. Journal of Animal and Plant Sciences 24: 1246-1251.

Vanhove AC, Vermaelen W, Panis B, Swennen R and Carpentier SC, 2012. Screening the banana biodiversity for drought tolerance: Can an *in vitro* growth model and proteomics be used as a tool to discover tolerant varieties and understand homeostasis. Frontiers in Plant Science 176: 1-10.

Wani SH, Sofi PA, Gosal SS and Singh NB, 2010. *In vitro* screening of rice (*Oryza sativa* L.) callus for drought tolerance. Communications in Biometry and Crop Science 5: 108-115.