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Research Article

Effect of salinity and drought stress on morphological and biochemical properties of two Iranian fenugreek (*Trigonella foenum-graecum*) populations

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Abstract

In this study, micro propagation of two Iranian fenugreek populations and their morphological and biochemical responses to salinity and drought stresses in *in vitro* culture condition were conducted using factorial experiment in a completely randomized design in three replications. Different explant type (terminal bud, cotyledon and epicotyledon explant) were cultured in MS medium contain different concentration of plant growth regulators such as kin (0, 0.5 and 1 mg / l) and 2,4-D (0.5, 1 and 2 mg / l). Murashige and Skoog (MS) medium supplemented with 1 mg/l kinetin and 2 mg/l 2,4-D showed the highest callus proliferation rate per explants in both populations. The highest amount of callus volume was obtained from the explants of the terminal bud. Proliferated calli from terminal bud explant were green and yellowish, from cotyledon were yellowish to white with soft texture, and the cotyledons were greenish and compact. The results of salinity stresses with different concentrations of sodium chloride (0, 70 and 120 mM) and drought stress with polyethylene glycol (0, 5 and 10%) showed that both stresses decreased callus growth and increased total protein, proline, catalase, peroxidase and trigonelin content in both populations. Trigonelin measurement showed that 'Borazjan' papulation had higher trigonelin content, *in vitro*, than 'Ardestan' papulation.

Keywords: callus induction; drought stress; fenugreek; salinity stress; trigonelin

Introduction

One of the severely detrimental factors to the growth and yield of the all crops around the world is drought and salinity stress. Also, all plants during ontogeny, have different interaction with its surrounding environment; they come in contact with different abiotic components like water, light, temperature, soil and chemicals (Anjum *et al.*, 2011). *Trigonella foecum-graecum* L. (fenugreek) belongs to the Leguminosae family (Rezaian *et al.*, 2011) it is used as forage, vegetable, medicinal plant and also for some preservative purposes. Fenugreek root, seed, and shoot are an important metabolite source such as trigonelin, diosgenin, neotycogenin and yamogenin and have high demand in the steroid industry (Esmaeil and Rezaeinodehi, 2014). Trigonline

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or N-methyl nicotinic acid is a secondary metabolite derived from pyridine nucleotides (Mehrafarin *et al.*, 2010). Drought and salinity stress in plant can alter various physiological activities, cause oxidative damages in various cellular components of the cell, interrupt in membranes activity, impact on photosynthetic activity, nutrient imbalance, decrease or increase in reactive oxygen species (ROS) detoxification system, damage on biomolecules such as membrane lipid, proteins, enzymes and nucleic acid (Abeles and Biles,1991). The osmolytes accumulation such as glycine, betaine, proline, and trigonellin in cell is known to protect organisms against abiotic stresses via osmoregulation or osmoprotection. Fenugreek is containing trigonelline that plays an osmoregulatory role in abiotic stress. Osmoregulatory properties of trigonelline revealed that fenugreek is a tolerant plant to environmental stress by increasing the endogenous melatonin and trigonelline, as well as the general physiological responses (Tramontano and Jouve, 1997). Plant tissue culture is a valuable biotechnological tool for plant research in different field such as cell signalling, gene manipulation, cell and plant physiology, morphogenesis, deep understanding the molecular biology and crop improvement using biotechnology (Khawar *et al.*, 2004). Here we tried to establish a high-performance callus induction and growth method to facilitate the basic research and genetic breeding of this important fenugreek species. Also, this study aimed to study the effect of salinity and drought stress on morphological, biochemical and quantitative and qualitative properties of secondary fenugreek metabolites due to the nutritional and medicinal value of fenugreek in Bushehr province in tissue culture system was performed.

Materials and Methods

This study was conducted in College of Agriculture and Natural Resources of Persian Gulf University. In this study, two genotypes of fenugreek ('Ardestan' and 'Borazjani') were used. Seeds washed first under running tap water at least 30 min, surface sterilized in 70% alcohol for 1 min, rinsed twice with sterile distilled water, immersed in beaker containing a sodium hypochlorite for 20 minutes and then immersed three times in sterile water for 5 minutes. After surface washing, the seeds were cultured in MS medium with paper supplement. For callus regeneration, 2-week-old buds, cotyledons and epicotyledons explants were cultured in MS medium containing 2,4-D (0.5, 1 and 2 mg / l) and kin (0, 0.5 and 1 mg / l). Cultures were kept at 25± 2 °C, 16 h of illumination (2000 L of light illumination) and 8 h of darkness in the growth chamber. After 30 days, morphological traits were recorded. To investigate the effect of salinity (NaCl: 0, 70 and 120 mM) and drought stress (PEG 6000: 0, 5 and 10%) on callus, the best callus regeneration medium (MS + 2 mg /l 2,4-D + 1 Kin) was prepared. After subculture of calli originated from the terminal-bud explants, the small pieces of callus were transferred to the selected culture medium for salinity and drought stress experiment. After 30 days biochemical traits were measured. Two-week-old seedling buds were used in field conditions to compare trigonelin levels with *in vitro* conditions.

Determination of biochemical properties

Protein extraction and measurement

One gram of callus was abraded with 3 mL of 25 mM tris, acid hydrochloric acid buffer (pH 6.8) and 3% polyvinylpyrrolidone (PVPP) in mortar and then incubated at 4 °C for 13,000 rpm, centrifuge for one hour. The upper phase containing total protein was separated and the total protein content was measured by Bradford method using BSA bovine serum albumin as standard curve with spectrophotometer at 595 nm (Bradford, 1976).

Proline

Proline content of treated plant was determined as described by Bates *et al.* (1973). After the analyses, following equation: (g proline in extract/115.5) g^{-1} sample = mol g^{-1} FW) were used for calculation the proline concentration from a standard curve.

Enzyme assays

At the end of experiment, leaves were collected and frozen in liquid nitrogen and stored at 280 °C before enzyme extraction. ROS scavenger enzymes were extracted as described by Zhang and Kirkham (1996) with some modifications. All operations were carried out at 4 °C. Intact leaves were ground using mortar and pestle under liquid nitrogen in cold 50 mM sodium phosphate, pH 7.5, containing 250 mM sucrose, 10 mM KCl, 1 mM $MgCl_2$, 1.0 mM EDTA, 0.5 mM 0.1 mM dithiothreitol, phenylmethylsulfonyl fluoride, and 1% (w/v) polyvinylpolypyrrolidone in a 6:1 proportion (w/v). The homogenate was then filtered and centrifuged at 25,000 g for 20 min at 4 °C. Then solid ammonium sulfate (NH4)2SO4 added to the supernatant to make up 80% saturated solution and allowed to stir gently for several hours at 4 °C. After centrifugation (28,000g for 45 min at 4 °C), pellets, were resuspended in a small volume of 50mM sodium phosphate (pH 7.5) and used for enzyme assays. The activity of peroxidase was measured by determining the increase in absorbance at 470 nm for 2 min using procedure of Hildebrand *et al.* (1986) and Heng-Moss *et al.* (2004). One millilitre reaction mixture contained 2 µl of 30% hydrogen peroxide 60 µl of 18 mM guaiacol, 20 µl of 200 mM HEPES (pH 7.0), 117 µl of distilled water, and 1 µl of callus extract. Catalase activities (ε H_2O_2 = 39.4 mM^{-1} cm^{-1}) were assayed spectrophotometrically according to Zhang and Kirkham (1996) method by monitoring the change in A240 due to the decreased absorption of H_2O_2. The reaction mixture contained enzyme extract, 50 mMNa-P, pH 7.0, and 15 mM H_2O_2 (in 1 mL final volume). The reaction was initiated by addition of H_2O_2. Catalase activity was determined according to the method used by Aebi (1984) in which the disappearance H_2O_2 in a reaction mixture containing 0.3 mL 3% H_2O_2, 2.5 mL of 0.05 M phosphate buffer (pH 7), and 2.5 mL of plant extract is monitored by the decrease in absorbance at 240 nm.

Trigonelin measurement

Stress-induced calli and two-week-old seedlings of a field grown plant of both papulations were sent to the Karaj University (Jihad Institute for Medicinal Plants) for trigonelin measurement. Samples (0.2 g of dried calluses) were powdered and sonicated with 10 ml of methanol by ultrasonic bath for half an hour. The mixture was then centrifuged (5000 rpm) for 10 minutes. The supernatant was evaporated and the residue was dissolved in 2 ml of methanol and stored in a refrigerator (4 °C). Determination of trigonelin had done by Agilent 1260 Infinity series HPLC (Agilent Technologies, Santa Clara, CA) equipment and column (C18, 25 Cm x 4.6 mm ID, 5 µm). The mobile phase consisted of the ratio of acetonitrile: water, 10:90, respectively. The elution time and flow rates were 6 min and 1 ml/min, respectively. The absorbance of trigonelin detected at 263 nm. For detection of trigonelin, the standard solution (Sigma) was used. The Open Lab software was used to peak integration (Rongjie *et al.*, 2010).

Statistical analysis

This experiment was performed in factorial arrangement based a completely randomized design with 3 replicates. Data were analysed by SAS software, and new Duncan multiple range test ($p < 0.05$) was used for determining the differences among treatments.

Results and Discussion

Callus regeneration rate

According to the results of analysis of variance analysis, a highly significant effect of the explant type, papulation type and the PGRs on the callus regeneration was observed. There was no significant difference between the PGRs and papulation on callus regeneration (Table 1).

Table 1. Analysis of variance (ANOVA) of explant type, papulation type and the PGRs and their interactions on the callus regeneration of fenugreek

SOV	*df*	Mean Squares
Population	1	126/23 **
Explant type	2	133/52 **
Plant growth regulator	8	71/14 **
Explant type× Population	2	123/97 **
Explant type× growth regulator	16	5/20 **
Population × plant growth regulator	8	0/67 ns
Population ×Explant type× Plant growth regulator	16	3/38 **
Error	108	0/85
Total	161	
CV (%)		9.08

**and ns were significant at 1% probability level and non-significant, respectively

The most abundant callus proliferation rate was observed on MS supplemented with 1 mg·L^{-1} 2, 4-D, and 1 mg·L^{-1} Kin in 'Ardestan' population as well as on MS media supplemented with 2 mg·L^{-1} 2, 4-D, and 1 mg·L^{-1} Kin in 'Borazjan' population. The lowest number of callus proliferation rate was produced by epicotyl explants cultured on media supplemented with different concentration of 2,4-D without kin in both populations. Also, the same results were obtained in case of using the terminal bud explant of 'Borazjan' population (Table 2).

Table 2. Effect of explant type and different concentration of Kin and 2,4-D on callus regeneration in different *Trigonella foenum- graecum* populations

PGRs (mg/l)		Population					
		'Ardestan'			'Borazjan'		
Kin	2,4-D	Terminal bud	Epicotyledon	Cotyledon	Terminal bud	Epicotyledon	Cotyledon
0	0.5*	10/00 $^{h-k}$	4/67 ^{q}	7/33 $^{m-p}$	5/83 ^{pq}	5/17 ^{q}	7/50 $^{l-p}$
0	1	10/50 $^{g-j}$	6/00 ^{opq}	7/83 ^{lmn}	7/33 $^{m-p}$	5/33 ^{q}	7/67 $^{l-o}$
0	2	12/50 ^{ef}	6/17 $^{n-q}$	9/17 $^{i-m}$	8/67 $^{j-m}$	4/33 ^{q}	9/33 $^{i-l}$
0/5	0/5	12/00 ^{efg}	7/67 $^{l-o}$	10/00 $^{h-k}$	7/50 $^{l-p}$	8/00 ^{lm}	9/00 $^{j-m}$
0/5	1	13/00 ^{de}	9/17 $^{i-m}$	11/83 ^{efg}	8/00 ^{lm}	10/50 $^{g-j}$	9/17 $^{i-m}$
0/5	2	16/00 ^{ab}	9/00 $^{j-m}$	11/67 $^{e-h}$	9/17 $^{i-m}$	10/33 $^{g-j}$	12/50 ^{ef}
1	0/5	15/67 ^{bc}	7/83 ^{lmn}	9/17 $^{i-m}$	9/17 $^{i-m}$	9/00 $^{j-m}$	8/33 ^{klm}
1	1	17/33 ^{a}	9/00 $^{j-m}$	10/00 $^{h-k}$	10/00 $^{h-k}$	9/17 $^{i-m}$	10/00 $^{h-k}$
1	2	17/00 ^{ab}	12/00 ^{efg}	12/50 ^{ef}	11/00 $^{f-i}$	11/00 $^{f-i}$	14/33 ^{cd}
Mean		13/78 ^{A}	7/94 ^{D}	9/94 ^{B}	8/52 ^{C}	8/09 ^{CD}	9/76 ^{B}

*Means in a column followed by the same letter do not differ significantly at $p = 0.05$. Mean with uppercase indicate the interaction of papulation and explant type and lowercase indicate the interaction between plant growth regulator, papulation and explant type. Callus regeneration rate was numbered based on the amount of callus apparent volume including very small callus 5, small 10, medium 15, large 20 and very large 25.

Numerous reports indicate a direct influence of PGRs on the callus induction of different type of explant in fenugreek. Jamshidi *et al.* (2014) reported that Fenugreek leaf explant in MS medium containing 0.5 mg/l Kin and 1.5 mg/l 2,4-D had the highest callus growth and weight. Vaezi *et al.* (2015) also reported the highest callus induction and growth of fenugreek cotyledon explants with MS medium containing 0.5 mg/l Kin and 1.5 mg/l 2,4-D. Khadiga *et al.* (2014) obtained the highest amount of callus in fenugreek using cotyledon explants in B_5 medium supplemented with 2 mg/l 2,4-D. Mohayeb *et al.* (2013), realized that 2,4-D in combination with Kin (2 and 0.5 mg/L respectively) obtained the highest amount of callus regeneration in hypocotyledon explant. Bashir and Alaf (2016) reported the best combination for producing the highest callus weight in fenugreek plant was 1 mg/l 2,4-D and BA. These findings are consistent with the present study in

terms of Kin content but differ in 2,4-D concentration. In general, the results of the present work confirmed literature reports regarding the auxins such as 2,4-D to initiate callus formation in many herbaceous species (Taha *et al.*, 2009). Gorel (2001) found that 2,4-D had the highest impact on callus regeneration than other type of the auxins. It seems that the use of auxins in high concentration than cytokinins can increase the callus regeneration rate in fenugreek. In this experiment, different colors and textures of callus were observed according to the explant type. But there was not significant difference between papulation in various hormone treatments. The callus formed was soft and light green colored, compact and yellow green or olive colored, soft, friable and yellowish colored in apical bud, cotyledon and hypocotyl explant respectively.

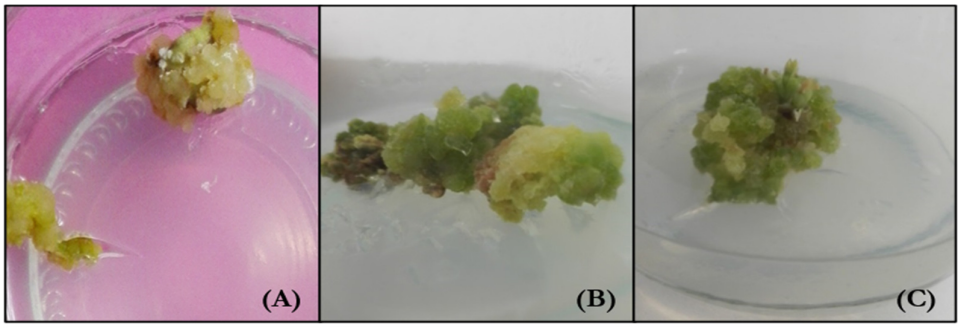

Figure 1. Colors and textures of callus on medium supplemented with different plant growth regulators (A) Soft and friable light green colored in apical bud explant (B) Compact yellow green callus in cotyledon (C) Soft yellowish callus in hypocotyl explant

Table 3. Callus growth performance of different explant of *Trigonella foenum-graecum* papulations under different PGRs treatments

PGRs (mg/l)		Population												
		'Ardestan'							'Borazjan'					
		Cotyledon		Hypocotyl		Apical Bud			Cotyledon		Hypocotyl		Apical Bud	
Kin	2,4-D	Color	Texture	Color	Texture	Color	Texture		Color	Texture	Color	Texture	Color	Texture
0	0/5	+	F	+++	F	++	C		+	F	+++	F	++	C
0	1	+	F	+++	F	++	C		+	F	+++	F	++	C
0	2	+	F	+++	F	++	C		+	F	+++	F	++	C
0/5	0/5	+	F	+++	F	++	C		+	F	+++	F	++	C
0/5	1	+	F	+++	F	++	C		+	F	+++	F	++	C
0/5	2	+	F	+++	F	++	C		+	F	+++	F	++	C
1	0/5	+	F	+++	F	++	C		+	F	+++	F	++	C
1	1	+	F	+++	F	++	C		+	F	+++	F	++	C
1	2	+	F	+++	F	++	C		+	F	+++	F	++	C

Yellowish (+++), Yellow- green (++), Green (+), F=Friable, C= compact

Salinity and drought stress effects on Trigonella foenum-graecum

Drought stress

In order to evaluate the response to salinity and drought stress of *Trigonella foenum-graecum*, at the cellular level in different papulations, callus was transferred to MS medium supplemented with 70 and 120 mmol l^{-1} NaCl and poly ethylene glycol (5 and 10% PEG). The relative growth rate of callus reached a maximum in the control treatment and at the presence of NaCl, growth was decreased with increasing NaCl concentrations (70 and 120 mmol l^{-1}). Also, no significant difference was observed within examined populations (Table 5). Noobar *et al.* (2010) reported that salinity stress on four Iranian fenugreek species showed that the fresh and dry weight of species decreased with increasing the salinity concentration. In another study, Kiani and Niknam (2015) reported that with increasing salinity, a remarkable decrease in callus growth was observed in both species of fenugreek. In the study of, as the concentration of sodium chloride increased, callus fresh weight in fenugreek decreased (Asam, 2011). The same results were reported in fenugreek (Zia *et*

al., 2010; Nam, 2011; Soheilikhah *et al.*, 2013). The results of these studies are in parallel with our present study. Decreasing the plant growth rate under salinity stress can be due to inhibition of cell division and proliferation or even cell death, water shortage, salt toxicity, nutrient imbalance, and impaired absorption, interfering with normal cellular processes, especially processes involved in energy production such as photosynthesis and respiration (Shibli *et al.*, 2007). The results in the Table 4 showed the impact of drought stress was significant increase in callus growth rate in treated calluses with PEG. The highest callus growth rate was observed in 'Ardestan' population in control treatment which had significant difference with other treatment. Also, with increasing PEG concentration, there was a significant decrease in callus growth in both populations, especially in 'Ardestan' population (Table 4). Kiani and Niknam (2015) reported that by increase in drought (mannitol) a marked decrease in callus growth in both species of fenugreek were observed. The results of these studies are consistent with the present study. Drought stress decreases growth by preventing cell growth and reducing cell division (Anjum *et al.*, 2011). Significance of the effect of callus growth on drought stresses confirms the existence of high genetic diversity among genotypes in terms of the traits evaluated which is in parallel with the report of Farhadi *et al.* (2014).

Table 4. Impact of salinity and drought stress on different on calli growth rate obtained from 'Ardestan' and 'Borazjan' populations of fenugreek

Population	NaCl (mM)				PEG (%)		
	0	70	120		0	5	10
'Ardestan'	22.22[a]	16/66 [bc]	13/89 [c]		22/22 [a]	15.00 [bc]	9.44 [de]
'Borazjan'	17.77 [b]	15.00 [bc]	13/89 [c]		17/77 [b]	12.22 [cd]	7.77 [e]

*Means in a column followed by the same letter do not differ significantly at $p = 0.05$.

Salinity stress

Metabolic activity within the callus was determined by measuring the proline content and catalase, peroxidase activity and total protein under salinity stress. Highest protein and proline content were obtained in stressed calli in media contain 70 mmol l^{-1} NaCl in 'Borazjan' population. In contrast, highest proline and protein content in 'Ardestan' population was observed in maximum salinity level. Protein and proline content of both stressed calli decreased respective to the control treatment. POX and CAT activities showed statistically significant differences ($P<0.05$) between stressed calli and respective control according to the intensity of salinity stress (Table 5). The highest POX and CAT activities was observed in media contain 120 mmol l^{-1} NaCl in both populations. However, these values were significantly in 'Ardestan' population ($P<0.05$) than their respective controls and 'Borazjan' population. Sarahi Nobar *et al.* (2010) reported that the protein content of *T. foenum-graecum*, *T. aphanoneura* and *T. tehranica* decreased under salinity stress and increased in *T. elliptica*. But, the proline content in *T. tehranica* and *T. elliptica* increased in 70 mmol NaCl. Also, the proline content in calli originated from *T. foenum-graecum* decreased in high concentrations of sodium chloride (120 ppm). Kiani and Nicknam's (2015) study showed that salinity reduced protein in calli of *T. foenum-graecum* and *T. aphanoneura* compared to control. Proline content of *T. foenum-graecum* was slightly decreased at 120 mM salinity and no significant change was observed in *T. aphanoneura* callus. In the present study, the amount of protein increased with increasing salinity compared to the control. But, in 'Borazjan' population at high salinity level a slight decrease was observed compared to salinity of 70 mM. The increase in protein content can be due to the induction of several proteins that are newly developed in response to salinity stress or are present at low concentrations, and when the plants are exposed to salinity, their concentration increases (Hall and Flowers, 1973; Levitt, 2015). In the present study, it seems that for the above-mentioned reasons, the amount of protein increased under salinity stress compared to control.

Proline content under salinity stress was increased compared to the control treatment. Proline content decreased in 'Borazjan' population at 120 mM salinity compared to the 70 mM salinity treatment. Accumulation of compatible solutes in the cytoplasm e.g. proline helps plant to maintain the osmotic balance and reduce the membrane damage. Compatible solutes do not impair normal physiological functions even if

accumulated at high concentrations (Pandey and Agarwal, 1998; Saneoka, 2004; Hayat *et al.*, 2012). Compatible solutes accumulation in cytosol and in organelles and its osmotic protection and association role with salt tolerance has been reported in higher plants. In the present study, it seems that the amount of proline can be used as a suitable indicator for assessing salt tolerance in fenugreek under in vitro conditions. Therefore, 'Borazjan' can be more resistant to salt stress than 'Ardestan' due to its high proline content. Kiani and Nicknam (2015) reported that catalase and peroxidase activity increased in the callus of two species of fenugreek under salinity stress (100 mM NaCl). These results are in parallel with the results of the present study. In this study, salinity in both fenugreek papulations increased the catalase and peroxidase activity compared to control treatment. Changes in the activity of antioxidant enzymes are one of the mechanisms that occur in plants to increase the plant tolerance in front of environmental stress. Catalase molecule can *convert* millions of hydrogen peroxide molecules to water and *oxygen* blocks free radical chain reactions (Rakmini, 2004) Therefore, it seems that calli were tolerate to this condition by increasing the activity of catalase and peroxidase.

Table 5. Impact of salinity stress on different biochemical parameters in calli obtained from 'Ardestan' and 'Borazjan' populations of fenugreek

Population	NaCl (mM)	Total protein (mg/g FW)	Proline (µmol/g FW)	CAT (µ mol/min.mg protein)	POX (µ mol/ min.mg protein)
'Ardestan'	Control	17.91 ^{c}	3/.23 ^{f}	0.002 ^{c}	0.06 ^{c}
	70	18.48 ^{c}	4.28 ^{e}	0.006 ^{a}	0.08 ^{ab}
	120	19.79^{c}	9.12 ^{c}	0.007 ^{a}	0.09 ^{a}
'Borazjan'	Control	13.07 ^{d}	7.13^{d}	0.003 ^{c}	0.06 ^{c}
	70	35.46 ^{a}	13.61^{a}	0.005 ^{b}	0.06 ^{c}
	120	31.69^{b}	12.63 ^{b}	0.006 ^{ab}	0.07 ^{bc}

*Means in a column followed by the same letter do not differ significantly at $p = 0.05$.

Kiani and Niknam (2015) studied two species of fenugreek under drought stress (0, 180- and 275 mM mannitol) and reported that the amount of protein and proline callus increased. In their study, *T. foenum-graecum* had higher proline content than *T. aphanoneura*. Navin *et al.* (2014) reported significant higher proline content than control on calluses from different explants of fenugreek under drought stress at different times and concentrations of mannitol. Similar results were reported by Salma *et al.* (2016) and Layegh Khoydaki *et al.* (2010), respectively, by studying different cultivars of *Pisum sativum* and *Salvia leriifolia* Benth under drought stress.

In the present study, by increasing drought stress and concentrations of PEG, proline content was increased in both populations compared to control. This proline accumulation under stress protects the cell from its adverse effects by osmotic adjustment of cytosol with that of the vacuole and external environments. Increase in proline content may relate to the decrease the incorporation of proline to proteins, synthesizing the anti-oxidants and enhancing activities of anti-oxidative enzymes or increasing expression of proline biosynthetic enzymes and decreasing proline degradation enzymes activity (Zhang, 1990). The obtained results in this study bring to surface the correlation between proline levels and drought tolerance in calli, obtained from different populations of fenugreek.

Table 6. Impact of drought stress on different biochemical parameters in calli obtained from 'Ardestan' and 'Borazjan' populations of fenugreek

Population	PEG (%)	Total protein (mg/g FW)	Proline (µmol/g FW)	CAT (µ mol/min.mg protein)	POX (µ mol/min.mg protein)
'Ardestan'	Control	17.91 ^{d}	3.23 ^{d}	0.002 ^{d}	0.06 ^{c}
	5	18.91 ^{d}	6.14 ^{c}	0.004 ^{c}	0.11^{a}
	10	23.87 ^{c}	9.43 ^{b}	0.005 ^{bc}	0.09 ^{b}
'Borazjan'	Control	13.07 ^{e}	7.13 ^{c}	0.003 ^{d}	0.06 ^{c}
	5	37.93 ^{b}	10.98^{ab}	0.006 ^{a}	0.07 ^{c}
	10	43.25 ^{a}	11.23 ^{a}	0.005 ^{ab}	0.06 ^{c}

*Means in a column followed by the same letter do not differ significantly at p = 0.05

Assessment of trigonelline content under drought and salinity stress

Results shown in the table (7) indicated that the drought and salinity stress had remarkable effect in decreasing trigonelline content of callus in all treatments. Totally, trigonelline content in both populations were decreased by increasing the severity of drought and salinity stress. The highest value of the compound trigonelline reached 1.261 mg/g dry weight in control treatment of 'Borazjan' and decreased compared with the salinity and drought stress treatments. The lowest trigonelline content among treatments were observed in drought stress treatments were in 10% PEG which gave 0.007 mg/g dry weight in 'Ardestan' population. Comparing the trigonelline content between field grown papulations of fenugreek plant with callus Trigonelline content revealed that field grown plant had higher trigonelline content. High trigonelline content from fenugreek calli compared to the in vivo condition presented by Ahmad *et al.* (2000) using different explant. They were recorded Trigonelline content in different part of plant including stem (0.21 mg/g), leave (0.45 mg/g) and root (0.29 mg/g) at in vivo condition. Whereas, the amount of trigonelline in calli as recorded in stem (0.61 mg/g), leave (0.3 mg/g) and root (0.21 mg/g).

Variable effects of sodium choloride and osmolytes such as manitiol and PEG on callus growth and secondary metabolite such as trigonelline content have been reported by different researchers (Berglund, 1996; Hussain and Aghlan, 2011; Cho, 2011; Bashir and Alaf, 2016, Bitarafan, 2018) and must have related research reported the positive effects of different stress on Trigonelline content increase. Whereas, the amount of recorded trigonelline content in calli in our study was lower than their respective control. In the other hand both studied stresses had negative effect on Trigonelline content. It seems this differ in results back to the genetic difference and seed origin of both populations.

Berglund (1996) reported that nicotine amide and its related metabolites, especially trigonellin, work and act as a carrier of the plant in response to oxidative stress. The osmolytes accumulation such as glycine, betaine, proline, and trigonellin in cell is known to protect organisms against abiotic stresses via osmoregulation or osmoprotection. Tramontano and Zhou (1997) found two-fold changes in trigonelline levels after induction of salinity in *Medicago sativa*. Further research to compare the effect of trigonelline and other osmotic regulators on cell cycle parameters in cultured chickpea (*Pisum sativum)* root cells showed that 10^{-4} to 10^{-7}mol/l trigonelline increased special molecules in the G_2 phase; proline is ineffective while beta-glycine was slightly effect in the accumulation of these substances in the G_2 phase (Tramontano and Jouve, 1997). In the present study, it seems that callus was resistant to the stress condition by enhancing biochemical substance including proline, protein and related enzymes. But, trigoneline content were decreased under stress conditions compared to control. Investigation of soybean trigonline content in saline and water deficit stress conditions showed that trigonline content were increased in young plants and decreased in the reproductive stage (Minorsky, 2002). In the present study, it is also possible that high trigonelline content in the field plant may be due to the growth stage and low amount of the trigonalin content in callus culture may related to the subculture effect, as reported for diosegene (Jamshidi *et al.*, 2014; Joanna *et al.*, 2015) .Further investigation on trigonelline is needed.

Table 7. Trigonelline content (mg/g) in calli obtained from 'Ardestan' and 'Borazjan' populations of fenugreek under salinity and drought stress

Population	Treatments					
	Culture condition		PEG		NaCl (mM)	
	Field grown	Control	5%	10%	70 (mM)	120 (mM)
'Ardestan'	0.852	0.313	0.014	0.007	0.068	0.018
'Borazjan'	0.494	1.261	0.809	0.806	0.494	0.394

Conclusions

In vitro tissue culture could be an important means of improving crop tolerance and yield through genetic transformation as well as by induced soma clonal variation. Therefore, it is important to devise an efficient protocol of callus proliferation to start *in vitro* selection for salt and drought stress tolerance, and to broaden opportunities for genetic manipulation of fenugreek (*Trigonella foenum-graecum*) through tissue culture, including trying various population and explants type. The results of this study indicated that MS medium contain 2 mg/l 2,4-D and 1 mg/l Kin showed a good callus induction and growth in cotyledon and hypocotyl and apical bud explants. Both salt and drought stress resulted to decrease in callus growth and increase in protein, catalase, peroxidase and proline content in both populations. Also, trigoneline content in both populations under salt and drought stress were decreased in comparison with control treatment.

Authors' Contributions

GHA and LK - designed the experiments, interpreted the data, performed statistical analysis. GA wrote the paper; AT - discussed the results, gave technical support; LK, MH, and GHA - Discussed the results, contributed to manuscript review and editing; Data curation; Formal analysis; Investigation and Methodology. All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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