The responses of potato cultivars to osmotic and temperature stresses under *in vitro* conditions

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Abstract

In order to investigate the responses of potato plantlets under *in vitro* conditions to osmotic and temperature stresses, a factorial experiment was carried out in a completely randomized design with three replications. Each replicate contained a glass jar with 5 explants grown in it. The first factor was water deficit at four levels of osmotic stress caused by polyethylene glycol 6000 including control, -0.5,-0.1, and -1.5 MPa and the second factor was temperature at three levels of 15, 25 and 35° C. Five potato cultivars, including 3 promising clones, 143, 301, and 306 as well as Marfona and Picasso cultivars were exposed to stresses in MS medium. Eight weeks after stress period, indicators such as fresh and dry weights of plantlets, electrolyte leakage, proline, catalase, phosphorus, and potassium concentrations were measured. The results of variance analysis indicated that the simple effects of factors as well as the interactions of the investigated factors at P≤0.01 had a significant effect on the study traits. Mean comparison showed that the temperature stress decreased the fresh and dry weights of plantlets and potassium concentration but increased electrolyte leakage, proline, catalase, and phosphorus concentration. On the other hand, osmotic stress reduced fresh and dry weight of plantlets, P and K, while proline, CAT, and electrolyte leakage increased. The combination of stresses lowered plantlets' resistance to stresses because osmotic stress of -0.5 MPa and higher, in combination with 35° C caused the explants to dry. Finally, results showed that Marfona cultivar was more tolerant to stress conditions than other genotypes. This seems to be the result of accumulating more proline and high antioxidant activity. On the other hand, clone 306 was more susceptible to stress than other genotypes and the plantlets 306 experienced more damage and dried.

Keywords: in vitro; osmotic stress; PEG; potato; temperature

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Introduction

Potato *(Solanum tuberosum)* is an important food source in many parts of the world. It is a critical crop in terms of food security (Birch et al., 2012). The crop is an essential source of starch, antioxidants, protein, vitamins, macro and micronutrients, polyphenols, carotenoids and tocopherols in the human diet (Brown 2005). Potato is susceptible to both drought and heat

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stresses (Monneveux et al. 2013). It is more sensitive to water deficit (Carr 1989). This sensitivity can be attributed to its small and shallow root system, which makes the plant ineffective for absorbing water (Gregory and Simmonds, 1992). Also, it is a cool season crop which is susceptible to heat stresses (Gelmesa et al., 2017).

Abiotic stresses caused by environmental factors could adversely affect the growth and development of crops (Mittler, 2006). In the last decade, lots of studies focused on the response of crops to a single stress (Siddiqui et al., 2015; Chew and Halliday, 2011). However, several abiotic stresses usually occur concurrently and crops are always subjected to a combination of different abiotic stresses in the field (Suzuki et al., 2014). Among the abiotic stresses, drought and heat are two critical threats to crop growth and sustainable agriculture worldwide (Lipiec et al., 2013; Awasthi et al., 2014). Heat stress is frequently associated with drought stress in field conditions (Ahuja et al., 2010), which makes necessary the study of crops responding to combined heat and drought stress. On a global basis, drought, in conjunction with coincident high temperature and radiation, poses the most important environmental constraints to plant survival and to crop productivity (Chaves et al., 2003).

Drought stress and high ambient temperature result in anatomical changes (Wahid et al., 2007; Zhang et al., 2005). In general, the changes include reduced size and damaged cells, closure of stomata and curtailed water loss, increased stomatal and trachomatous densities, and larger xylem vessels. Drought, due to its osmotic effect in natural and agricultural habitats can induce a wide number of responses ranging from growth inhibition and synthesis of some nontoxic compounds to increased osmotic potential of the cell and thus allowing metabolic processes to continue to enhance some antioxidant enzyme activities (Turkan et al., 2005). High temperature considerably affects anatomical structures not only at the tissue and cellular levels but also at the sub-cellular level. At the sub-cellular level, main modifications refer to the shape of chloroplasts, swelling of stromal lamellae, clumpy vacuoles that change the structural organization of thylakoids and form antenna depleted PS II, and thereby reduce photosynthetic and respiratory activities (Zhang et al., 2005).

One of the negative effects of stress in plants is the reduced production of fresh biomass and dry matter. Greater plant fresh and dry weights under water limited conditions are desirable characteristics. A common adverse effect of water stress on plants is the reduction in fresh and dry biomass production (Farooq et al., 2009). Water shortage causes fundamental problems for metabolic processes in plant cells and induces growth inhibition. The main effects of water deficit in cells are reduction of water potential, increased concentrations of compounds in the cell sap, decreased turgor pressure, and changes in structure and conformation of macromolecules (Smirnoff, 1993). Physiological and biochemical processes such as photosynthesis, respiration, translocation of assimilates, ion uptake, carbohydrate metabolism, and nutrient metabolism are disrupted (Jaleel et al. 2009). According to Anjum (2011) during the period of drought stress, the number of stomata decreases, the amount of fresh and dry weights in the plant's organs is affected, and fresh and dry weights of the plant are reduced. By induction of drought stress on potato under *in vitro* medium its fresh and dry weight and number of leaves are shown to decrease (Najafzadeh and Ehsanpour, 2012). In another study on potatoes, increasing drought stress decreased potato plantlets' fresh and dry weights (Golestani Kermani et al. 2014).

Plasma membrane is the first site that suffers under stress conditions (Levitt et al. 1980). Cell membrane stability can be considered as criterion for tolerance to drought and heat stresses (Nazari Nasi et al. 2012). As a result of damage to cell membranes, leakage of materials increases and ultimately the stability of cell membranes reduces and so the cell death occurs (Blume and Ebercon, 1981). Malondialdehyde (MDA) production due to the destruction of cell membranes is a response of plants to environmental stresses (Munns 2002). The main injuries under high temperatures include protein denaturation and increased fluidity of membrane lipids and inactivation of enzymes, reduced synthesis and degradation of proteins, and defaults in membrane integrity (Howarth, 2005;

Kozlowska, 2007). Under drought and heat stresses, it was reported that ion leakage increased in peppermint (Hokmabadi et al. 2017) and colocynth (Hassandokht et al. 2017).

Osmotic adjustment through the accumulation of cellular solutes, such as proline, has been suggested as one of the possible means for overcoming osmotic stress caused by the loss of water (Caballero et al., 2005). Proline is a non-protein amino acid formed in most tissues subjected to water stress (Singh et al., 2000). Proline accumulation may result from further degradation of proteins and sensitivity of cell to drought stress. Proline also serves as a sink for energy to regulate redox potentials, a hydroxyl radical scavenger (Sharma and Dietz, 2006), a solute that protects macromolecules against denaturation and as a means for reducing acidity in the cell (Kishor et al., 2005). Proline accumulation in the cell cytoplasm decreases osmotic potential and increases water absorption under stress conditions (Anjum et al. 2014). High levels of proline enable the plant to maintain low water potentials causing the accumulation of compatible osmolytes that allows additional water to be taken up from the environment, thus buffering the immediate effect of water deficit within the organism (Moussa and Abdel- Aziz, 2008). Bohenert and Shen (1999) reported that there is a positive correlation between proline accumulation and adaptation to osmotic stress conditions under drought stress. Proline increased due to stress in grape's explants under *in vitro* culture (Mehri et al., 2015), Pear (Javadi et al., 2004) and wheat (Gholipour and Ebadie, 2017).

Drought and high temperatures induce significant alterations in plant biochemistry and metabolism. Under drought stress, plants deal with the stimulated production of reactive oxygen species (ROS), (e.g. singlet oxygen, superoxide radical, hydrogen peroxide, hydroxyl radical (Liu and Huang, 2000) that cause membrane injuries, protein degradation, enzyme inactivation and thus induce oxidative stress (Zlatev and Lidon, 2012). To be able to endure oxidative damage under adverse conditions, plants possess enzymatic antioxidants such as SOD, POX, APX, CAT, and GR (Bowler et al., 1992; Smirnoff, 1996). These antioxidant enzymes are reported to increase under various environmental stresses (Hernandez et al., 1995). For example, comparatively higher activity has been reported in tolerant species than in the sensitive ones (Bor et al., 2003; Shalata et al., 2001). Catalase is the most important antioxidant that decomposes hydrogen peroxide in the form of O_2 and H_2O and reduces the amount of H_2O_2 in the medium (Aebi 1984). Increased activity of catalase enzyme due to stress was reported in tomato (Nasibi, 2011) and almond (Zokaee et al., 2014).

Nutrition uptake from soil is directly related to the status of soil water because by reducing the soil moisture, nutrition diffusion flow decreases from soil to root surface (Arndt et al., 2001). Drought generally reduces nutrient uptake in plants and concentrations of mineral nutrients in plant tissues (Fageria et al., 2002). Generally, nutrient uptake by plants grown in soil is greatly influenced by several factors including climate and water stress (Alam, 1999). The reduction in nutrient uptake by plant under drought stress is due to the reduced transpiration and impaired active transport as well as membrane permeability resulting in the reduced root absorption power (Tanguilig et al., 1987). Water stress affects nutrient transportation to the root and root growth. However, crop species and genotypes within a species are known to differ in their ability to take up nutrients under drought stress conditions (Garg, 2003). Inorganic nutrients including P and K ion play multiple essential roles in plant mechanisms (Ashraf et al., 2008). Use of nutrients such as K reduces the toxicity of ROS by increasing the concentration of antioxidants, e.g. superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) in plant cells (Waraich et al., 2012). A decrease in the concentration of K ion results in membrane damage and distortion of ionic homeostasis (Kozlowska, 2007). Drought stress reduced the uptake of P and K in peanut (Kulkarni et al., 1988). It was reported that in rapeseed, drought stress significantly decreased K content in leaves and roots (Amoubeigi and Razavizadeh, 2013). Enrichment of tissue with P in white clover (Singh and Sale, 2000) improved drought toleration ability. Similarly, K supplementation proved helpful in mitigating the adverse effects of water stress in peanut and sorghum (Umar, 2006).

Table 1
Characteristics of parents of potato promising clones under study

Genotype	*Male*	*Female*
143	Caeser Veriety	Promising Clone of S. tuberosum × S.stoloniferum
301	Promising Clone of S. tuberosum	Luka Variety
306	Luka Variety	Promising Clone of Caeser Veriety × S.stoloniferum

Polyethylene glycol (PEG)-6000 as a reliable marker in laboratory condition is used to test the effects of drought stress in plants. PEG acts as a non-penetrating osmotic agent resulting in the increased solute potential and blockage of absorption of water by root system (Ranjbarfordoei et al., 2000). Regarding the fact that drought stress and temperature (both high and low temperature) affect all aspects of plant growth, the present study was conducted under *in vitro* conditions to evaluate the combination of stresses on potato varieties, the purpose of the study was to investigate the response of potato genotypes to these types of environmental stresses, due to the removal of other factors on stress response in order to select the most tolerant cultivar.

Material and Methods

This research was carried out in Tissue Culture and Micro-Propagation Laboratory, Faculty of Agriculture, Mohaghegh Ardabili University, Ardabil, Iran on three promising clones, numbers 143, 301, and 306 (Table 1) and Picasso and Marfona varieties, which were obtained from the collection of Agricultural Research Station of Ardabil. The factorial design was carried out based on a completely randomized design with three replications and each replicate included a glass container containing five explants. The first factor was osmotic stress caused by PEG - 6000 at four levels of control, - 0.5, -1, and -1.5 MPa (Michel and Kaufmann, 1973) and the second factor, temperature at three levels of 15, 25, and 35 °C were applied to the genotypes under study.

In order to prepare plant explants for rooting under *in vitro* conditions and applying stresses, small cuttings containing 2 cm-long stems were prepared from plants cultivated in the greenhouse. The explants were cultured after sterilization in a rooting medium containing MS medium including 8 g l^{-1} agar and 30 g l^{-1} sucrose.

After rooting and proper growth of the explants, single-node explants of 2 cm length were transferred to the final culture medium containing polyethylene glycol. Glass containers containing explants were transferred to the growth chambers that were set at their temperature as 18-22 °C, with a light period of 16 hours of brightness and 8 hours of darkness. PEG was added to the medium by using of the diffuse technique (Taji et al. 2008). Eight weeks after stress, samples were taken to measure the traits.

Traits measurement

Fresh and dry weight

Eight weeks after stress, plantlets were weighed (fresh weight) with a digital balance (accuracy 0.001), then samples were placed in an oven set at 70 °C for 24 hours. After 24 hours, the explants were weighed again to obtain plantlet dry weight (mg).

Electrolyte leakage

Lutts et al. (1995) method was used to measure electrolyte leakage. The samples were rinsed twice in distilled water and then put in falcons containing 10 ml of distilled water and placed on a shaker at room temperature (25 °C) for 24 hours. The primary electrical conductivity (EC_1) was measured. In the next step, the samples were autoclaved and the secondary electrical conductivity (EC_2) was measured after cooling and reaching room temperature. The percentage of electrolyte leakage was calculated by the following equation.

Table 2
Variance analysis of different PEG concentrations and temperature stresses on studied traits in potato varieties under *in vitro* conditions

S.O.V	df	Mean Squares						
		FW (mg)	DW (mg)	EL (%)	P (g 100 g^{-1} DW)	K (g 100 g^{-1} DW)	Pro (µmol g^{-1} FW)	Cat (µmol min^{-1} g pro)
Osmotic stress (A)	3	1506077.62**	4752.55**	656.3**	7.62**	5245.42**	261324.6**	4032.97**
Heat stress (B)	2	8024.32**	217.55**	11554.11**	3.33**	1632.56**	306471.32**	4931.15**
Genotype (C)	7	43384.19**	183.74**	866**	0.96**	158.40**	8354.99**	731.54**
(A) × (B)	6	5483.09**	39.93**	3889.14**	1.83**	679.12**	156057.39**	2292.78**
(A) × (C)	12	40186.64**	154.91**	400.7**	0.16**	39.37**	2637.63**	124.25**
(B) × (C)	8	49011.06**	223.02**	395.5**	0.16**	26.22**	1841.64**	21.99**
(A) × (B) × (C)	24	43559.27**	192.74**	571.56**	0.14**	26.03**	2609.88**	99.12**
Error	120	395	1.55	0.56	0.017	6.67	9.54	1.43
	-	14.21	11.59	1.99	11.74	11.32	2.13	6.58

**: significant at P≤0.01; C.V.: Coefficient of Variation

$$EL\% = EC_1/EC_2 \times 100 \quad (1)$$

Proline assay

Proline was measured by Bates et al. (1973) method and the absorbance was read at 520 nm with a spectrophotometer and was measured in µm g^{-1} fresh weight (FW).

Catalase activity

The catalase enzyme was measured using Aebi method (1984) and the rate of variation at 240 nm wavelengths was obtained by using spectrophotometer in µm min^{-1} g protein.

Nutrition contents

In order to measure the amount of elements in plant tissue, samples were placed in an oven set at 70 °C for 24 hours. Then from each sample, 0.05 g dried tissue was poured into a 100 ml glass jar. Three ml of concentrated nitric acid was added to each sample and kept for 48 to 72 hours in the laboratory. The containers were warmed slowly under the hood. The appearance of white smoke and the loss of acidic solution was a sign of the end of digestion. The extracts were plain paper and the volume of the remaining distilled water solution reached 50 ml. Then, the concentration of potassium was determined by a flame photometer. Phosphorus was also measured by colorimetric method using a spectrophotometer at 470 nm. Using the standard curve, potassium and phosphorus were calculated in terms of g/ 100 g dry matter.

Statistical Analysis

Statistical significance was assessed at P≤0.01 using ANOVA and means were separated by LSD test (P≤0.05) with the help of SAS 9.1 software. Means and standard deviation were calculated from 3 replicates**.** Figures were also drawn by Excel.

Results

Results (Table 2) showed that the effects of simple factors and the interactions were significant on all measured traits at P≤ 0.01.

Fresh weight

Study of control treatment showed that in the absence of osmotic stress, plantlets had the highest fresh weight at 25 °C (without temperature stress) compared to the other temperatures while at 15 and 35 °C, it was less than 25 °C. At 15 °C, the plantlets had the least amount of fresh weight. Among the cultivars, Marfona cultivar showed the lowest fresh weight in the control treatment compared to the other cultivars while in the same conditions 306 had the highest amount of fresh weight. By increasing PEG concentration, under osmotic stresses, the seedling fresh weight decreased and in -1.5 MPa osmotic stress the minimum fresh weight was seen at 15 °C. Compared with other varieties 143 showed low fresh weight while other cultivars were in the next category, and the 301 retained its fresh weight at high level. It was obvious that in the absence of temperature stress (25 °C), both the low and high fresh weights of the plantlets were the highest and the temperature stress had

a negative effect on fresh weights. The simultaneous effects of stress caused the plantlets to lose more fresh weight and some plantlets reduced their fresh weight and theirs fresh weight reached zero as they dried. Accordingly, osmotic stresses of -0.5 MPa and higher in combination with temperature at 35 °C caused the plantlets to die (Fig. I). Results showed that in control, fresh weight had positive and significant correlations with dry weight and electrolyte leakage at P≤0.01 while it had a positive and non-significant correlations with P, K, and proline concentrations and finally its correlation with catalase enzyme was negative and non-significant (Table 3). At the osmotic stress level of -0.5 MPa, fresh weight had positive and significant correlations with dry weight and electrolyte leakage at P≤0.01 while it had positive but not significant correlations with P,

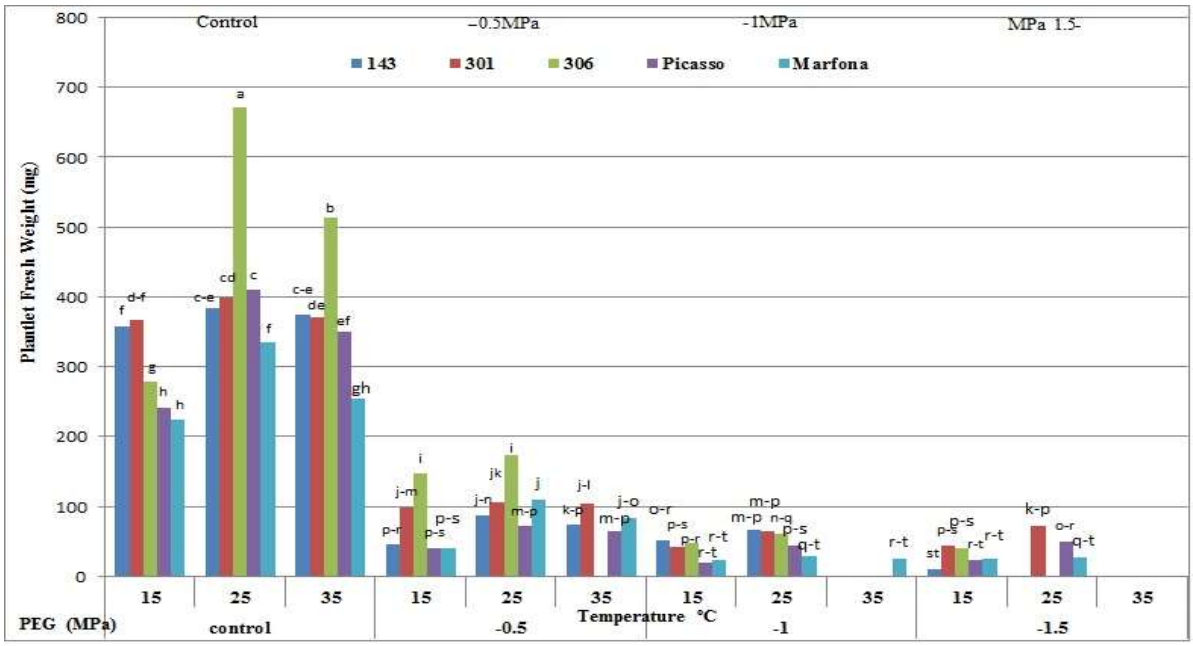

Fig. I. The effect of PEG concentrations and temperature stresses on fresh weight of potato varieties under *in vitro* conditions

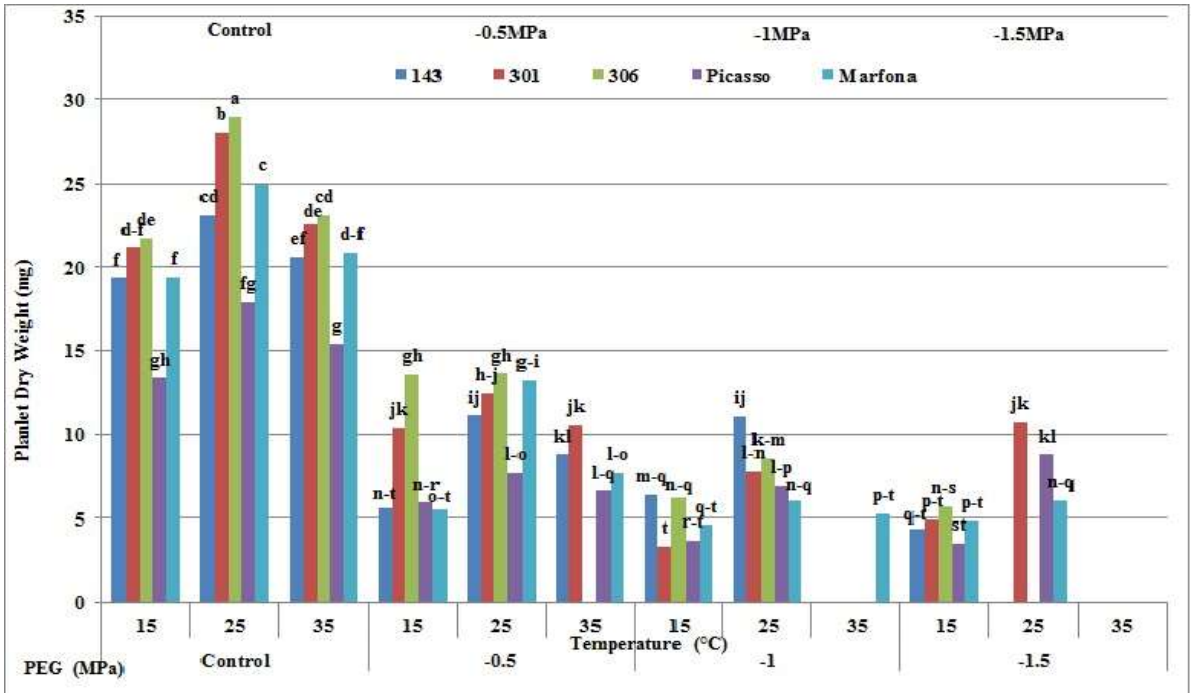

Fig. II. The effect of PEG concentrations and temperature stresses on dry weight of potato varieties under *in vitro* conditions

Table 3
Correlation coefficient of studied traits at the control treatment in potato cultivars under *in vitro* conditions

	Fresh Weight	*Dry Weight*	*El*	*P*	*K*	*Proline*	*CAT*
Fresh Weight	1						
Dry Weight	0.631**	1					
El	0.721**	0.626**	1				
P	0.030^{ns}	0.307^{ns}	0.076^{ns}	1			
K	0.253^{ns}	0.424^{ns}	0.368^{ns}	-0.554*	1		
Proline	0.271^{ns}	0.601*	0.596*	0.515*	0.275^{ns}	1	
CAT	-0.401^{ns}	-0.151^{ns}	-0.340^{ns}	0.341^{ns}	-0.055^{ns}	0.460^{ns}	1

**: significant at P≤0.01 *: significant at P≤0.05 ns: non- significant

Table 4
Correlation coefficient of studied traits in -0.5 MPa PEG in potato cultivars under *in vitro* conditions

	Fresh Weight	Dry Weight	El	P	K	Proline	CAT
Fresh Weight	1						
Dry Weight	0.933**	1					
El	0.607*	0.707**	1				
P	0.447^{ns}	0.583*	0.843**	1			
K	0.468^{ns}	0.603*	0.883**	0.927**	1		
Proline	0.376^{ns}	0.527*	0.914**	0.899**	0.961**	1	
CAT	0.255^{ns}	0.423^{ns}	0.764**	0.803**	0.882 **	0.943**	1

**: significant at P≤0.01; *: significant at P≤0.05; ns: non- significant

Table 5
Correlation coefficient of studied traits in -1MPa PEG in potato cultivars under *in vitro* conditions

	Fresh Weight	Dry Weight	El	P	K	Proline	CAT
Fresh Weight	1						
Dry Weight	0.935**	1					
El	0.851**	0.880**	1				
P	0.731**	0.765**	0.943**	1			
K	0.805**	0.796**	0.968**	0.966**	1		
Proline	0.766**	0.820**	0.985**	0.965**	0.975**	1	
CAT	0.659**	0.771**	0.936**	0.937**	0.925**	0.977**	1

**: significant at P≤0.01

K, proline, and catalase (Table 4). In osmotic stresses of -1 and -1.5 MPa, the fresh weight of plantlet had a positive and significant correlations with dry weight, P, and K concentrations, proline, and catalase at P≤0.01 (Tables 5 and 6).

Dry weight

In relation to dry weight, the study of plantlets cultivated in the control treatment showed that in the absence of osmotic stress, the dry weight of plantlets was the highest at 25 °C (the absence of temperature stress) and if the temperature was higher or lower than 25 °C, dry weight decreased. Results also suggested that at 35 °C, the plantlet dry weight was higher than 15 °C. In terms of cultivars, the results showed that 306 had the highest and Picasso cultivar had the lowest dry weight. By increasing the concentration of PEG in the medium, in osmotic stress, dry weight of the plantlets was decreased and the lowest amount was observed at -1MPa in 301 while in -1.5 MPa, dry weight was more than -1 Mpa. It seems that osmotic stress had the highest effect on dry weight and decreased, because the absence of temperature stress (temperature 25 °C) did not affect the dry weight of the plantlets. The simultaneous effect of stresses on seedlings reduced the amount of dry weight so that the concentration of PEG, -0.5 MPa and higher in combination with 35 °C dried the plantlets (Fig. II).

Table 6
Correlation coefficient of the studied traits in the -1.5MPa PEG in potato cultivars under *in vitro* conditions

	Fresh Weight	Dry Weight	El	P	K	Proline	CAT
Fresh Weight	1						
Dry Weight	0.965**	1					
El	0.819**	0.881**	1				
P	0.884**	0.938**	0.966**	1			
K	0.862**	0.898**	0.976**	0.971**	1		
Proline	0.809**	0.885**	0.986**	0.983**	0.980**	1	
CAT	0.709**	0.798**	0.939**	0.927**	0.940 **	0.970**	1

**: significant at P≤0.01

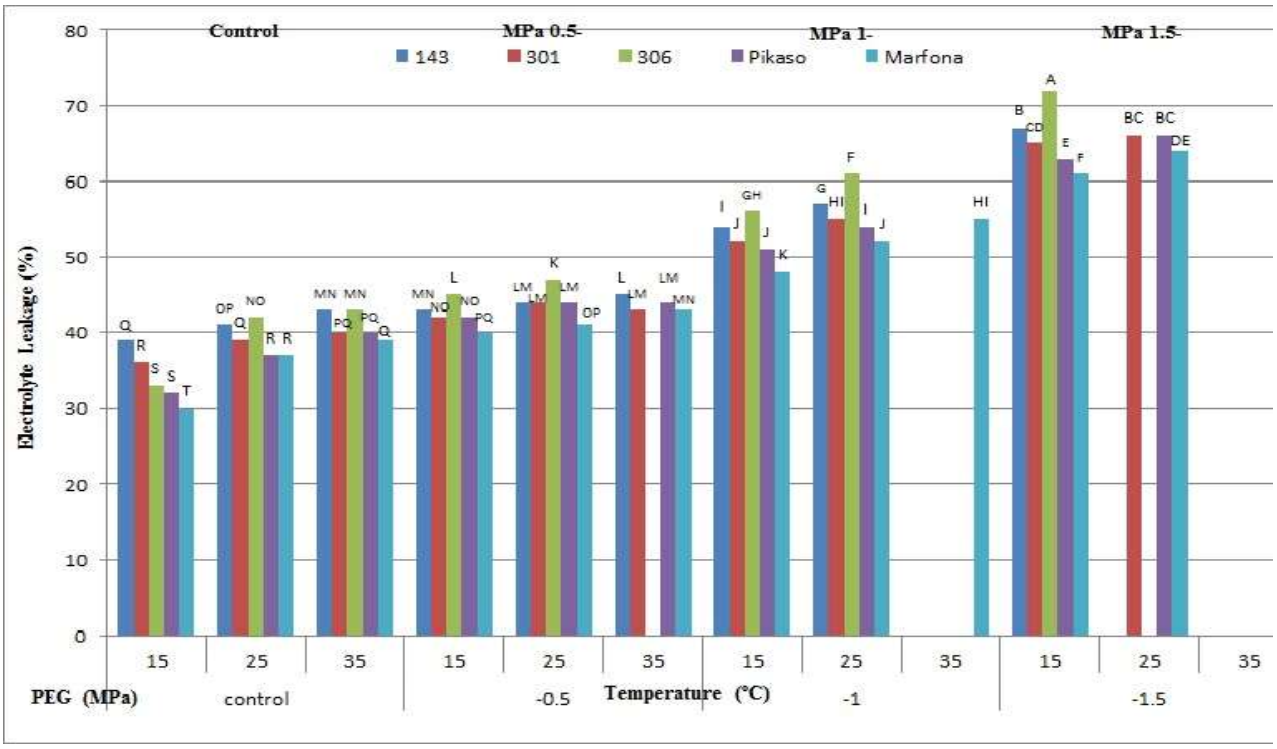

Fig. III. The effect of PEG concentrations and temperature stresses on EL percentage of potato varieties under *in vitro* conditions

In the control treatment, the results showed that dry weight of the plantlets had positive and significant correlations with electrolyte leakage and proline (P≤0.01) while there was a positive and non-significant correlation with P and K, and a negative and non-significant correlation with catalase enzyme (Table 3). Under the osmotic stress level of -0.5 MPa, dry weight of the plantlets showed a positive and significant correlation with electrolyte leakage (P≤0.01), a positive and significant correlation with P, K, and proline (P≤0.05), and a positive but non-significant correlation with catalase (P≤0.05) (Table 4). Under osmotic stresses of -1 and -1.5 MPa, seedling dry weight had a positive and significant correlation (P≤0.01) with electrolyte leakage, P, K, proline, and catalase (Tables 5 and 6).

Electrolyte leakage

In the control without PEG, the comparison of the temperatures showed that the temperature stress increased the instability of the membranes. Accordingly, 15 °C compared to 25 °C and 35 °C showed a lower ion leakage rate and increasing the temperature in the absence of osmotic stress resulted in the membrane

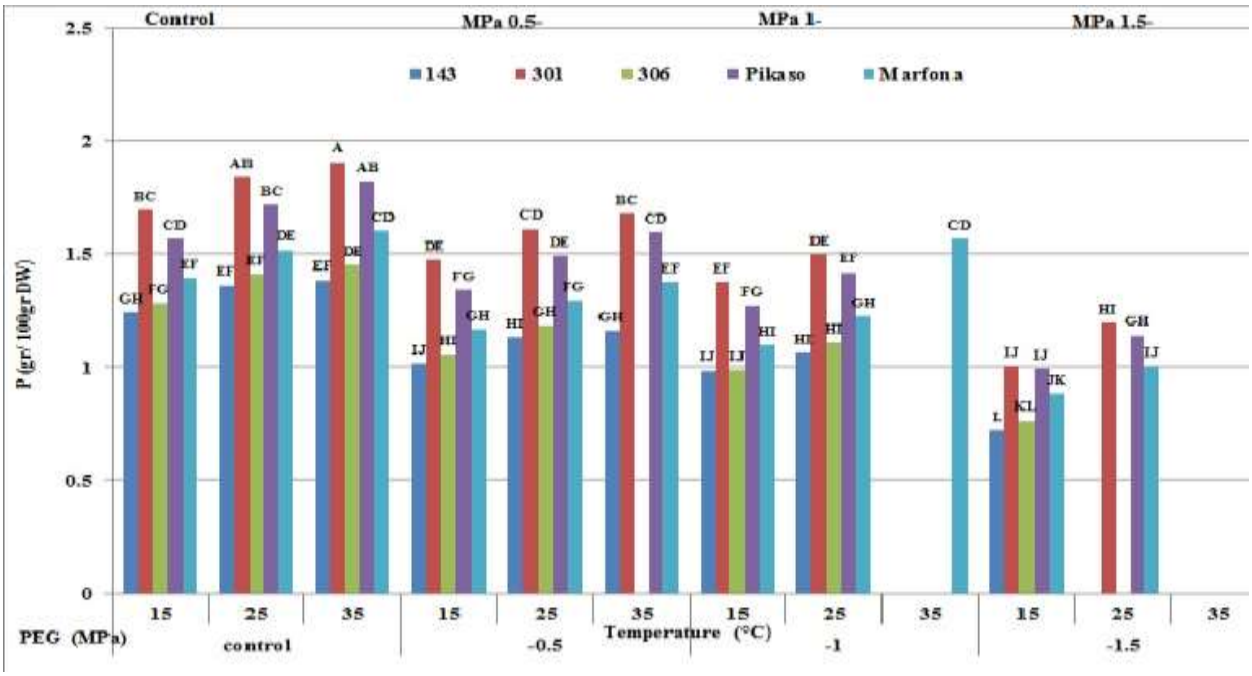

Fig. IV. The effect of PEG concentrations and temperature stresses on P content of potato varieties under *in vitro* conditions

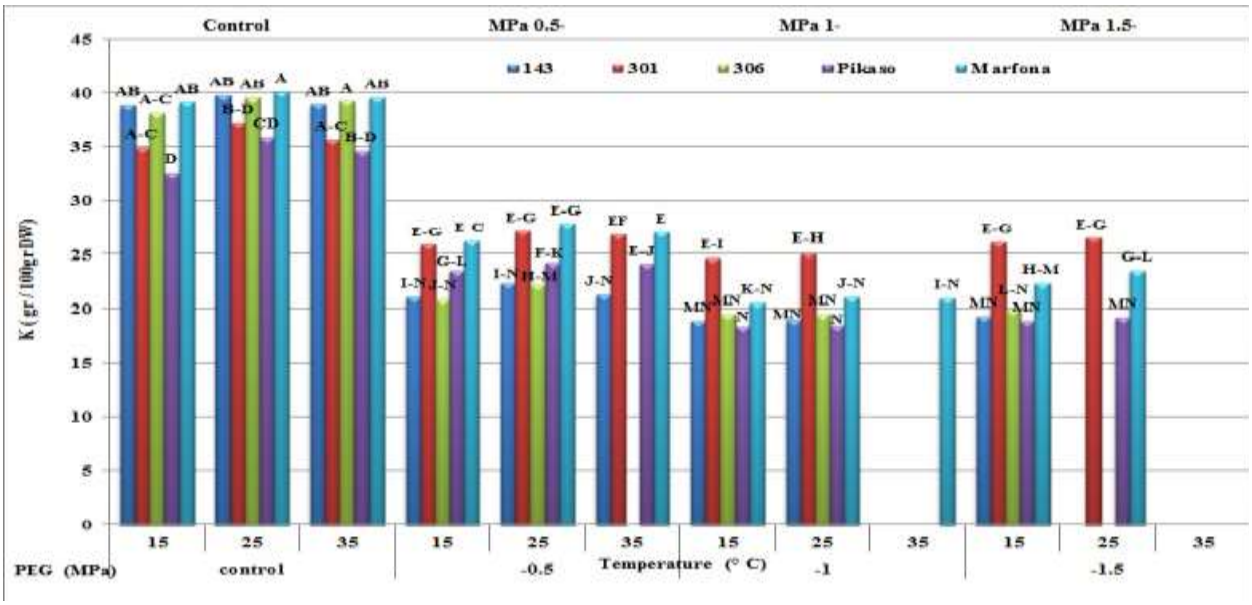

Fig. V. The effect of PEG concentrations and temperature stresses on K content of potato varieties under *in vitro* conditions

instability. However, Marfona was distinguished by having a lower percentage of membrane instability than all other cultivars at all three temperatures, and it was able to tolerate the conditions while the 306 had more ionic leakage, and was susceptible to stress. Comparison of ion leakage at three concentrations of PEG in the absence of temperature stress (25 °C) showed that increased PEG concentration increased significantly instability of membranes, so that its percentage at 25° C and PEG concentration -1 MPa increased to 65% in clone 306. On the other hand, when combination of stresses was applied on plantlets, the amount of electrolyte leakage was significantly increased, and plantlets that were exposed to osmotic stress of -0.5MPa and higher and 35 °C dried and among the varieties, clone 306 showed the highest susceptibility and its seedlings dried. It can be concluded that plantlets have better conditions at 15° C where they can tolerate

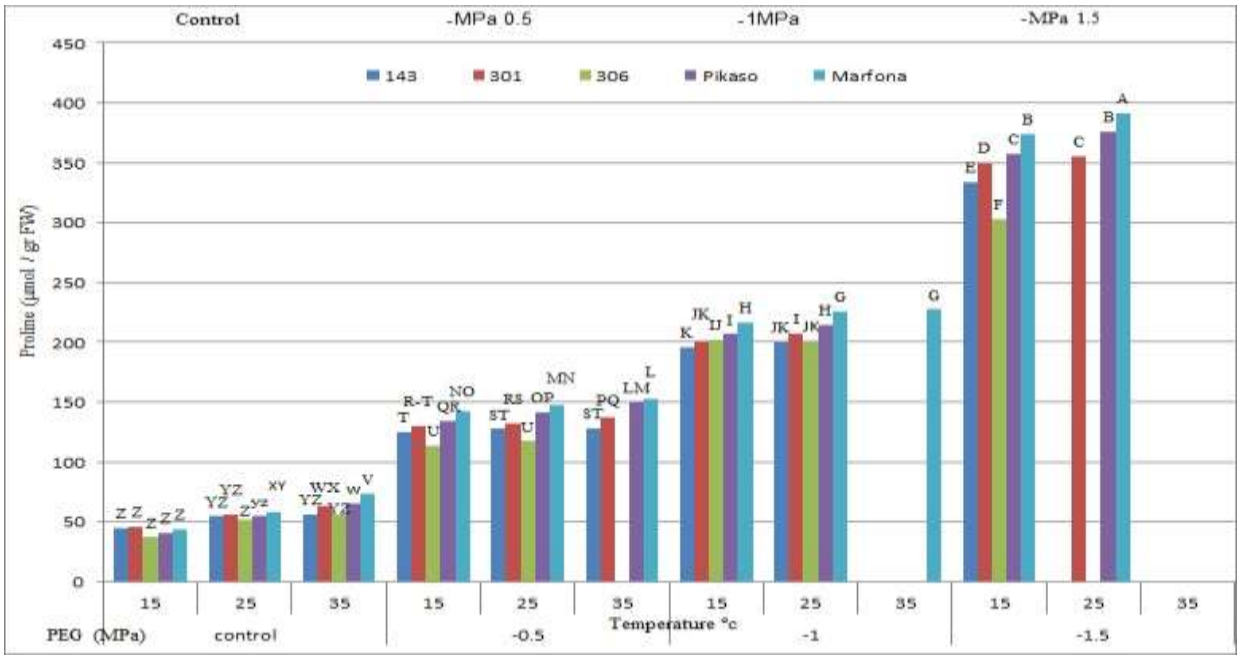

Fig. VI. The effect of PEG concentrations and temperature stresses on proline accumulation in potato varieties under *in vitro* conditions

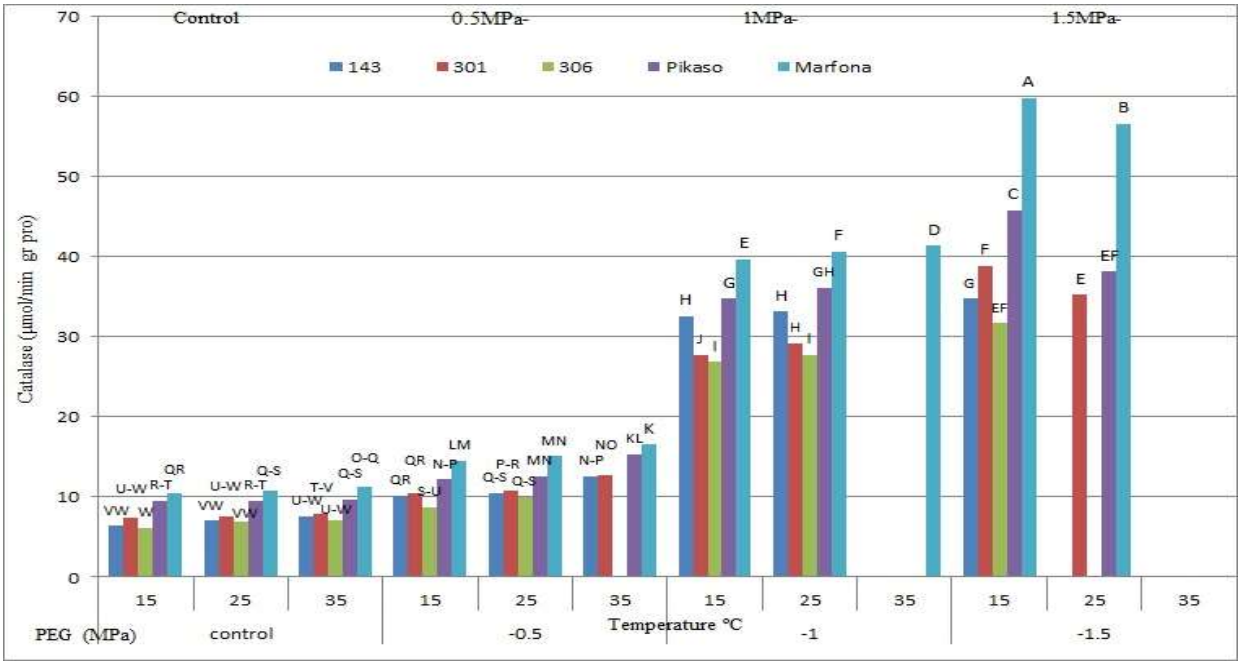

Fig. VII. The effect of PEG concentrations and temperature stresses on catalase accumulation in potato varieties under *in vitro* conditions

the highest concentrations of PEG and do not dry (Fig. III). In the control treatment, results showed a positive and significant correlation between electrolyte leakage and proline (P≤0.05) and a positive and non-significant correlation with P and K, and negative and non-significant correlation with catalase enzyme (Table 3). Under osmotic stresses of -0.5, -1 and -1.5 MPa, electrolyte leakage had a positive and significant correlation with P, K, proline, and catalase (P≤0.01) (Tables 4, 5 and 6).

Phosphorus

In relation to P, the study of three temperatures in control treatment showed that the increase in temperature in the absence of osmotic stress caused an increase in the amount

of P. The comparison of the temperatures indicated that P was high at 35 °C compared to 15 and 25 °C. Results showed that among the cultivars, 301 had the highest amount of P while 143 had the minimum level of P in its tissues. Increasing the concentration of PEG in the medium resulted in the reduction of P in plantlets and this decreasing of P at -1.5MPa reached to maximum content. Clone 143 was distinguished by the minimum amount of P from other cultivars, whereas in the same condition, clone 301 performed better and showed more P. On the other hand, the combination of stresses showed a decrease in the content of P and therefore, at 15 °C and -1.5 MPa, less P was absorbed. Also, the osmotic stress of -0.5 MPa and higher and 35 °C dried the plantlets. However, the cultivars based on the amount of P were 301, Picasso, Marfona, 306 and 143, respectively. (Fig. IV) Results showed that in control treatment, there was a negative and significant correlation between P and K at P≤0.05 while a positive and significant correlation was found with proline P≤0.05 and a positive and non-significant correlation was found with catalase (Table 3). There was a positive and significant correlation between P and K, proline, and catalase in the osmotic stresses of -0.5, -1 and -1.5 MPa at P≤0.01 (Tables 4, 5 and 6).

Potassium

The study of the temperatures in the control treatment showed that the amount of K in plant tissues was higher in the absence of osmotic stress at 25 °C in comparison with the other temperatures and its content decreased at 15° C and 35 °C. Moreover, at 35 °C this was greater than at 15 °C. On the other hand, results showed that among the cultivars, Marfona absorbed more K followed by 306, 143, 301, and Picasso in that order. Different concentrations of PEG in the medium up to -1 Mpa decreased K content of plant tissues and the lowest K content was found in Picasso cultivar while 301 showed higher K content than other cultivars. However, increase in PEG concentration to -1.5 MPa resulted in increased K content. Initially, the simultaneous effect of stresses decreased the K content of the tissues and the lowest values were observed at 15 °C and -1 MPa while osmotic stresses of -0.5 MPa and more along with the temperature of 35 °C caused the plantlets to dry (Fig. V). In control treatment, correlation coefficients showed that K had a positive and non-significant correlation with proline but the correlation was negative and non-significant with catalase (Table 3). In the osmotic stresses due to the addition of PEG to the medium, K showed a positive and significant correlation with proline and catalase at concentrations of -0.5, -1.0, and -1.5 MPa at the probability level of 1% (Tables 4, 5 and 6).

Proline

In the absence of osmotic stress in the medium, the results indicated an increase in proline accumulation as temperature was raised. Accordingly, the comparison of the three temperatures showed that 35 °C had a higher proline accumulation than 15 °C and 25 °C, indicating that the presence of more proline resulted in the plantlets' tolerance to the high temperature stress in the control treatment, and Marfona showed the highest proline accumulation. This was followed by Picasso, 301, 143, and 306 cultivars in that order. Increasing the concentration of PEG in the medium increased the amount of proline production in the plantlets, so that the plantlets could resist stress conditions, thus the highest amount was seen in the -1.5 MPa and 25 °C in Marfona. When osmotic and temperature stresses were applied in combination, results showed that more proline was produced so that different concentrations of PEG for plantlets at 15 °C were tolerable because of the presence of proline and the plantlets survived while 35 °C and the concentration of -0.5 MPa and higher caused the plantlets to dry in other varieties (Fig. VI). Results showed that proline had a positive and non-significant correlation with catalase in control (Table 3). Under osmotic stresses, at concentrations of -0.5, -1.5, and -1.0 Mpa, proline had a positive and significant correlation with catalase at P≤0.01(Tables 4, 5 and 6).

Catalase

Regarding the catalase enzyme, the comparison of the studied temperatures in the absence of PEG showed that the temperature was effective in increasing the activity of the enzyme; however, 35 °C compared to the other temperatures led to higher enzymatic activities and the plantlets could tolerate stress conditions. This was more pronounced in the Marfona at 35 °C while 306 showed a high sensitivity due to low enzymatic activity. Investigating different concentrations of PEG showed that increasing PEG concentration in the absence of temperature stress (25 °C) caused a significant increase in the enzyme activity, so that at -1.5 Mpa and 25 °C, Marfona plantlets were able to tolerate stress while in other cultivars the enzyme activity decreased. In this condition, cultivars 306 and 143 could not survey and dried. The simultaneous effect of both stressors on the plantlets increased the enzyme activity and increasing catalase activity is one of the strategies for coping with stresses. Seedlings that were exposed to osmotic stress at 35° C were not tolerant and the plantlets dried. It seems that the seedling tolerance threshold for catalase activity was in 15 °C higher than other temperatures, and the plantlets were able to resist the highest concentration of PEG at 15 °C (Fig. VII).

Discussion

Crops respond to abiotic stresses with various modifications at morphological, cellular, physiological, biochemical and molecular level (Siddiqui et al., 2015; Zhou et al., 2015). Plants have evolved acclimation and adaptation mechanisms to cope with water deficit, including avoidance, escape from stress, and dehydration tolerance of the protoplast. During water deficit, many physiological and biochemical processes are disturbed (Deikman et al. 2012; Juenger, 2013).

In order to screen the potato genotypes under *in vitro* culture, Zhang and Donnelly (1997) used length and fresh and dry weights of stem and root. Water stress is one of the limiting factors in plant growth that greatly affects cell division and expansion. In comparison with control, drought stress significantly reduces the fresh and dry weights of the plant due to alteration in some plant characteristics, which include changes in chlorophyll content, damage to photosynthetic structure, damage to PS II, inhibition of photochemical activity and Kelvin cycle enzymes, increase in chlorophyll fluorescence (Dulai et al., 2006), inhibiting the synthesis of photosynthetic pigments and reduction of stomatal conductance (Hasanuzzaman et al., 2013), reduction of carbon fixation, limitation of water and nutrients uptakes (Ashraf and Foolad, 2007), change in CO_2 assimilation, transpiration, reduction of leaf area, acceleration of the leaf senescence (Wahid and Rasul, 2007), limitation of production, and distribution of photosynthetic assimilates in plants. Reduction in the activities of source and sink takes place under heat stress which greatly affects the growth (Taiz and Zeiger, 2006). High-temperature stress reduces root growth, number, and mass (Huang et al., 2012), which affects the growth of aboveground tissue by restricting the supply of water and mineral nutrients, affecting production of hormones synthesized in roots and transported to shoots, and altering sink-source relationships between shoots and roots (Huang et al., 2012; Wahid et al., 2007; Hao et al., 2012). According to Rasheed et al. (2011) heat stress reduced the bud fresh and dry weights. Under *in vitro* condition of four Iranian grape varieties, Mehri et al. (2015) indicated that fresh and dry weights of explants reduced due to water stress. In another experiment on cherries at different concentrations of PEG, the results showed that the use of PEG reduced the fresh and dry weights of shoots, shoot length, and the plant water content (Sivritep et al., 2008).

Drought and heat stresses caused oxidative damage through the production of free oxygen radicals in cells, and these free radicals attacked proteins, lipids, and nucleic acids and also decreased cell membrane stability, causing the cytoplasmic leakage. Munns and James (2003) confirmed that in drought conditions, cell membrane integrity was damaged and MDA increased by production of reactive oxygen species and membrane lipid peroxidation. Masoumi et al. (2010) reported that the drought stress led to damage in the integrity of the cells and cell membranes via disrupting the function of reactive oxygen species' scavenging systems. Jiang and Hung (2001) reported that under drought and heat stresses, MDA concentration increases due to

the rise in lipid peroxidation and oxidation of membrane fatty acids. Increasing electrolyte leakage is a sign of damage to membrane stability, which is probably the result of oxidative stress. Due to the unsaturated fatty acid degradation by ROS, compounds such as malondialdehyde are produced that cause toxicity to the cell. The accumulation of malondialdehyde under stress conditions increases the permeability of the plasma membrane. Ion leakage increases, which gives an indication of the amount of oxidative damage (Golen et al., 2006). Under drought and heat stresses, cell membranes lose their stability; therefore, the membrane stability is determined by the evaluation of ion leakage. Heat stress markedly limits membrane stability, photosynthesis, respiration, and water balance, and also disturbs primary and secondary metabolites in plants (Hemantaranjan et al., 2014, Ding et al., 2016). It seems that cell membrane stability under stress conditions is related to the synthesis of heat shock proteins and the features of the photosynthetic system, including enzymes, and the cell membrane that maintains its stability during stress play an important role in tolerance to drought and heat (Bewley, 1979). In a study by Nouri et al. (2016) on ten potato cultivars, Marfona cultivar showed more membrane stability than other cultivars, and its ion leakage rate was lower than other varieties. Hassani Moghdam et al. (2016) observed that water stress in pomegranate leaves increased the amount of electrolyte leakage. Also, under *in vitro* culture of almond, cell membrane stability was significantly higher in resistant cultivars and increasing PEG concentration in the medium increased ion leakage in the leaves of sensitive explants (Karimi et al., 2013).

Drought stress often limits the uptake of nutrients by the plant. The absorption of nutrition by plants under water deficit conditions is reduced due to reduced transpiration, impaired active transmission, and permeability of the membrane, thereby reducing root absorption capacity (Levitt 1980). Many important nutrients are absorbed by roots along with water, the drought conditions limit the movement of these nutrients via diffusion and mass which leads to retarded plant growth (Barber, 1995). Also, with decreasing of soil moisture, the rate of nutrition release from the soil to the root absorbance level is reduced. Plant root system efficiency may also be reduced as a result of soil moisture deficit (Alam, 1999). Heat stress reduces the number, mass, and growth of the roots which ultimately limits the supply of water and nutrients to the plant shoots (Wahid et al., 2007; Huang et al., 2012).

Phosphorus plays its role as an energy storage for oxidation and compounds require energy (as adenosine diphosphate) and can provide the energy needed for metabolism. In addition, P plays an important role in carbonation (Nematollahi et al., 2013). Under environmental stress conditions, the deficiency of P can disrupt the synthesis of phosphate carbohydrate and nucleotides in the structures of DNA and RNA molecules. Reduction of soil moisture decreases P uptake (Kafi et al., 2010) and can reduce its solubility and availability and can decrease transpiration and the growth and development of the root system (Hopkins and Huner, 2009). In the case of water stress, the rate of P release from soil to root surface is restricted compared with other nutrients, because phosphate ion attach to clay particles and becomes less available to the plant (Marschner, 1995). Investigating the response of bean plant to drought showed that under stress conditions, the ability of P uptake by the plants roots was low. The reason is the impact of PEG on the absorption and transfer of P in the vessels and stems of plants (Hadidi, 1999). Reduced phosphorus levels due to water stress was reported in plants such as chamomile (Pirzad et al., 2015), and sunflower (Nematollahi et al., 2013).

Potassium is an essential cytoplasmic nutrition and is often considered as an important element in stress conditions because of its role in osmotic regulation and its competitive effect with sodium. K plays an important role in regulating the potential of plant cells and also activates many enzymes involved in photosynthesis and respiration (Wind et al., 2004). K is also involved in the synthesis of protein, cooperation in the transfer of amino acids, neutralization of organic acids, cell division and expansion, and resistance to disease and adverse environmental conditions such as drought and cold (Fernando et al., 1992). It plays a role in regulating CO_2 absorption and elimination by controlling the closure of stomata. In addition, the activity of many enzymes that use

K^+ as co-factor is reduced (Gimmenz and Frere, 1986). Cakmak (2005) reported that if the plant had favorable K status, it could reduce the production of ROS in the plant by reducing the activity of NADPH oxidase and keeping stable the electron transfer path. It was reported that heat stress reduced the tissue levels of K^+ and Ca^{2+} (Rasheed et al., 2011). Based on the results of research on two sunflower cultivars, stress reduced the amount of K (Nematollahi et al., 2013). Under in vitro culture of canola, increasing the stress affected roots and shoots and decreased their K content (Amoubeigi and Razavizadeh, 2013). Meanwhile, Tarahomi et al. (2011) observed that the increase in drought stress caused a significant decrease in K content of roots, but increased K content in leaves.

Proline accumulation is a common response to a wide range of environmental stresses, including low water levels and high and low temperatures in plants. Osmotic adjustment is a mechanism to maintain water relations under osmotic stress. It involves the accumulation of a range of osmotically active molecules/ions including proline, soluble sugars, sugar alcohols, glycine betaine, organic acids, calcium, potassium, chloride ions, etc. Under water deficit and as a result of solute accumulation, the osmotic potential of the cell is lowered, which attracts water into the cell and helps with the maintenance of turgor. Potato responds to drought and other stresses by accumulating proline which functions as an osmo-protector, osmo-regulator, and ROS scavenger (Benavides et al., 2000). Increased heat stress leads to the overproduction and accumulation of various organic and inorganic osmolytes. These osmolytes protect the plants from stresses by cellular osmotic adjustment, detoxification of ROS, protection of biological membranes, and stabilization of enzymes/proteins (Bohnert and Jensen, 1996; Verbruggen and Hermans, 2008). Plant tolerance to unfavorable conditions particularly water deficit, has been associated with proline accumulation, which may represent a water loss regulatory mechanism by reducing cell water potential (Fumis and Pedras, 2002) and may also be a biochemical marker of metabolic alterations generated by different types of stress (Lima et al., 2004). Drought induced accumulation of proline caused by both activations of its biosynthesis and the inactivation of its degeneration is considered to act as an osmoprotectant, a ROS scavenger, and a molecular chaperone stabilizing the structure of proteins, thereby protecting cells from damage caused by stress (Szabados and Savoure, 2010). Proline protects the protein structure of plant cells. It also helps to maintain the cell membrane through interaction between phospholipids. In addition, they are used to eliminate hydroxyl radicals or as sources of nitrogen and energy storage (Vendruscolo et al., 2007).

It is generally accepted that under conditions of water deprivation or high temperature, proline accumulation serves as a defense mechanism against stress challenge by acting as a compatible solute (Hare and Cress, 1997). The metabolite analysis has shown that under stress, the precursor for proline biosynthesis L-glutamate-semi-aldehyde and L-proline itself began to increase. Significantly, L-glutamate-semi-aldehyde and L-proline are respectively the products of two enzymes, pyrroline-5-carboxylate synthetase (P5CS), and pyrroline-5-carboxylate reductase (P5CR) which play major roles in the proline biosynthetic pathway (Delauney and Verma, 1993). In some plant species such as potato, proline plays a major role in regulating osmotic pressure. It can be said that the sensitivity of the varieties to the stress is due to the lack of proline formation in the sensitive variety, and it can be concluded that proline in the potato plant is considered as an indicator of resistance to stress and plays a key role in osmotic regulation in stress conditions, causes a resistance to stress, and reduces the damage of osmotic stress (Amini et al., 2017). Najafzadeh and Ehsanpour (2012) observed that by increasing drought stress in potatos under *in vitro* culture, the amount of proline in aerial part of plants increased significantly compared to the control. Masoudi Sadaghiani and Amini Dehghi (2016) reported that drought stress increased the accumulation of proline in potato. In addition, a positive relationship has been reported between tolerance and proline accumulation in almond (Zokaii et al., 2014).

Oxidative damage is usually a subsequent stage of most abiotic stresses in plants. Environmental extremes including drought, low

and high temperatures, salinity and heavy metals create oxidative stress (Navari-Izzo and Rascio, 1999; Sgherri et al., 1996, Rasheed et al., 2011). In order to cope with the oxidative stress, plants usually rely on the antioxidant defense which can be either enzymatic or non-enzymatic. Enzymatic defense is usually considered as the most effective (Farooq et al., 2008). To be able to endure oxidative damage under such an adverse condition, plants possess antioxidants such as ascorbic acid, glutathione, α- tocopherol, carotenoid, flavonoids and enzymes such as SOD, POX, APX, CAT (Bowler et al., 1992; Smirnoff, 1996). These antioxidant enzymes and metabolites are reported to increase under various environmental stresses (Acar et al., 2001). Also, comparatively higher activity has been reported in tolerant species than in the sensitive ones (Shalata et al., 2001; Bor et al., 2003). During exposure to stress conditions, reactive oxygen species (ROS) are one of the major causes of cellular damage in plants. ROS is generally accumulated in cells when plants are exposed to environmental stresses. Catalase enzymes are important antioxidant defense systems in plants (Sairam and Srivastava, 2002). CAT is only present in peroxisomes and it is indispensable for ROS detoxification during stress when high levels of ROS are produced (Mittler, 2002). CAT either directly scavenges the ROS or protects plants indirectly by managing non-enzymatic defense (Anjum et al., 2011). CAT eliminates H_2O_2 by breaking it down directly to form water and oxygen (Winston, 1990; Smirnoff, 1993). It prevents ROS accumulation, and thus protects the plant against lipid peroxidation of membrane systems and oxidative damage under drought stress (Mafakheri et al., 2011). It has also been reported that plants are able to withstand against heat stress by increasing the antioxidant enzymes activity (Sariri et al., 2011). Masoudi Sadaghiani and Amini Dehghi (2016) showed that in potato, the activity of CAT was highest under severe stress. Also, increases in the CAT contents were reported in tomato (Sanchez-Rodriguez et al., 2010) and cucumber (Amini et al., 2017). Razavizadeh and Shahriari (2017) confirmed the increase of CAT activity due to stress in sorghum. In a study carried out by Zokaii et al. (2014) on several species of almond it was shown that in some species, under the highest levels of drought stress, the activity of CAT in leaf was the highest.

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