

Physiological and Morphological Responses of Almond Cultivars under *In Vitro* Drought Stress

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Abstract

In this study, physiological and morphological responses of five almond cultivars to drought stress were investigated under *in vitro* conditions. Plantlets from five commercial almond cultivars (Supernova', 'Tuono', 'Sahand', 'Ferragnes' and 'Shahroud 21'] were established in MS medium containing 0.5 mg per liter BAP and then subcultured in MS proliferation medium containing 1 mg per liter BAP. Plantlets were exposed to four different levels of polyethylene glycol containing 0, 2, 4 and 6 percent, respectively, which is equivalent to 0, -0.14, -0.36 and -0.66 bar of water potential as drought stress levels during four weeks. This study was carried out as a two-factor factorial experiment in a completely randomized design with three replications, each consisting of two jars, each contained two explants. At the end of the stress period, physiological indicators of stress, including leaf relative water content (RWC), proline content and ion leakage of leaves and morphological indicators, including plantlet height and number of developed leaves, were measured. The results showed that drought stress increased ion leakage and proline content, while it reduced the RWC, plantlet height and number of developed leaves. According to these results, 'Supernova' and 'Tuono' were less affected by drought stress compared to other cultivars, while 'Shahroud 21' and 'Ferragnes' were more affected than others. 'Sahand' also showed an intermediate performance compared to other cultivars.

Keywords: Almond, Drought stress, Polyethylene glycol, Tissue culture.

Introduction

Identification of drought tolerant germplasms in plants is very important, and it is one of the important breeding purposes in applied researches for most of the crops (Romero *et al.*, 2004). Wild and native germplasms of almond are a valuable genetic source for important physiological characteristics such as drought tolerance that can be identified and used for breeding programs (Sorkheh *et al.*, 2012).

Most of the plants have specific mechanisms for

drought tolerance and increase the water use efficiency. Drought tolerance mechanisms are widely known in agricultural crops but fewer studies have been conducted in fruit trees. In addition to morphological mechanisms, other mechanism such as osmotic regulation and changes in root's volume, fresh and dry weight to aerial organs will affect the whole plant (Rieger and Duemmel, 1992). Despite the research in this subject, the relative importance of each one of these mechanisms has not

been fully specified.

Understanding the mechanisms of drought tolerance in the plant will ease decision making in the matter of irrigation management and improve the performance of efficient genotypes in the drought stress conditions (Torrecillas *et al.*, 1996; Isaakidis *et al.*, 2004).

Alizadeh (2006) showed that measuring relative water content is the most reliable method for the measurement of water content in plant tissues and therefore, it is being used more than any other method. Plants use different ways to maintain their physiological activities under dry conditions. For example, many plants maintain their turgescence by increasing the concentration of ions in cells of leaves, roots and other organs in response to drought (Morgan, 1984). Rasouli (2000) stated that grape cultivars that have the ability to absorb more water or have more water-use efficiency under drought conditions and can keep their RWC level higher in their organs will have more resistance to drought stress.

Chartzoulakis *et al.*, (2002) reported that drought stress causes reduction in relative water content (RWC) in leaves of avocado and only 10% reduction in the the (RWC) causes a significant reduction in water potential of leaves. Research by Romero *et al.* (2004) showed that the variation range for RWC in drought stress conditions is approximately 95-73% and daily and seasonal changes in the RWC level of almond leaves is lower than water potential. The experiments of Alarcon *et al.* (2002) showed that GF677 rootstock is more resistant to drought stress than Garrigues seedlings due to the prevention of water loss through transpiration and keeping the leaves water potential during dry conditions. Turkan *et al.* (2005) considered that comparing to sensitive cultivars, the RWC level in the leaves of resistant cultivars were not affected by drought stress, while in sensitive cultivars, the RWC was reduced up to 10% in 14 days. Torrecillas *et al.* (1996) stated that high levels of RWC in studied almond cultivars are specific characteristics of xeromorphic

plants. Teulate *et al.* (1997) stated that RWC is a very good indicator of plant water condition and can be considered as an index for selection of drought tolerant species.

Turkan *et al.*, (2005) observed that the RWC level in leaves of drought resistant species was not affected by drought stress. Research on mulberry plants showed that an increase in drought stress causes a significant reduction in the RWC level (Ramanjulu *et al.*, 1998). Kramer (1983) indicated that there is a significant and strong relation between RWC and the photosynthesis rate. Savee *et al.* (1990) reported that ion leakage of leaf samples of kiwi fruit increased in response to drought stress and similar results were shown under greenhouse and in vitro conditions.

Sairam *et al.* (2002) reported that genotypes that are sensitive to salinity show a significant reduction in their cell membrane stability index. Saneoka *et al.* (2004) stated that plant cells which are resistant against osmotic changes during drought stress have more cell membrane stability. Karimi *et al.*, (2012) reported that in vitro drought stress significantly increases the ion leakage of fig's leaf samples and plant's drought resistance has an inverse relationship with leaf samples ion leakage under drought stress condition.

Nowadays, some physiological parameters such as proline are being used to evaluate drought stress. According to Taylor opinion (1996), proline is the most important organic molecules that accumulate in plant tissues during environmental stress. Also, Sofu *et al.* (2004) stated that proline is one of the active amino acids in osmotic regulation which has an important role in development and regulating of the osmotic pressure in plant cells. One of the most important physiological effects of proline, regardless of its role in osmotic regulation, is its role in protection of membrane structure and proteins (Blum 1986; Kavikishore *et al.*, 2005).

The reason for accumulation of proline inside of plants under stress is due to an increase in synthesis of

proline (because of reduction in oxidation of glutamate) and a reduction of proline consumption for protein formation (due to suspension of plant growth) (Larher *et al.*, 2009).

Al-Khayri and Al-Bahrany (2004) and Sivritepe *et al.* (2008) reported a direct relationship between proline accumulation in plant's tissues and drought tolerance. Proline's role in the elimination of active types of oxygen molecules under tension condition was reported by (Turkan *et al.*, 2005). According to research by Zamani *et al.* (2002) on almonds, when the drought stress is severe enough, the accumulation of proline increases. Sircelj *et al.* (2005) did not observed a constant algorithm in the amount of proline in cultivars of apple, which are under heavier stress levels. Previous reports have shown that as PEG increases (which leads to a more intensive stress), the amount of proline significantly increases in resistant cultivars of date palm seedlings (Naidu *et al.*, 2005).

Sivritepe *et al.* (2008) and Molassiotis *et al.* (2006) showed that a reduction in the number of developed leaves can be considered as a defense mechanism against drought tolerance, which helps plants absorb less light and therefore has less transpiration surface. This can be due to less water demand of cell division compared to the cell elongation process. Also, results of an experiment carried out on almond seedlings showed that by increasing the time between irrigations, vegetative indicators, such as plant height, shoot diameter, leaf area and dry weight of aerial parts, decreased significantly (Mousavi *et al.*, 2009). In another experiment carried out by Jalilimarandi *et al.* (2011), water deficit tension affected all vegetative indicators of three cultivars of grape. According to these results, as the soil moisture decreases, shrub height, shoot diameter, number of developed leaves, leaf area, dry and fresh weight of leaf and shoot and dry weight of plant decreased. The aim of this research was to study of physiological responses of almond cultivars under in vitro drought stress.

Materials and Methods

Plants for this research were obtained from current year shoots of five years old late blooming cultivars including 'Ferragnes', 'Shahroud 21', 'Tuono', 'Sahand' and 'Supernova'. These plants were taken from almond cultivar from the Seed and Plant Improvement Institute located in Meshkin-Shahr of Karaj, Iran. The Research process was started in May 2014.

For sterilization, shoots were placed under running tap water for an hour and submerged in 70% ethanol for 30seconds and then submerged in 2.5% sodium hypochlorite solution for 20 minutes. Shoots were rinsed three times in sterile distilled water and then explants with 15–20 mm length (single or two nodes) were prepared and individually transferred to jars containing 15 ml of MS (Murashige and Skoog, 1962) basal medium. The medium were supplemented with 30 gL⁻¹ sucrose, 0.5gL⁻¹ BAP and 9 gL⁻¹ agar. The pH of the media was adjusted to 5.7 ± 0.05 with HCl 0.1 N or NaOH 0.1 N prior to sterilization by autoclave at 121°C for 15 minutes. Cultures were maintained at 25±3°C and 16:8 h photoperiod. After 30 days, uniform developed explants were excised and transferred to the same medium that contained 0.1 mg L-1 BAP. After 30 days, uniform developed explants were selected and transferred to the MS media containing different concentrations of poly ethylene glycol (PEG) (0, 2%, 4 and 6%). Plant growth regulator was not added to these media. The incubation conditions were the same as described above. The experiment was carried out as a factorial experiment based on a completely randomized design (CRD) with two factors and three replications per treatment and two jars per replication. The first factor was the different concentrations of PEG (0, 2, 4, and 6%), and the second was different almond cultivars.

After 30 days, at the end of experimental period, plantlet height, number of developed leaves, ion leakage, RWC and proline content of leaves of plantlets were measured. For measuring plantlet height, digital caliper

was used. Relative water content of explants' leaves (RWC) was measured using non broken leaf discs (Kirnak *et al.*, 2001).

Ion leakage was measured using the method described by (Sullivan *et al.*, 1979). Proline content was measured in 500 mg of leaf material via the method described by Bates *et al.* (1973).

Analysis of variance (ANOVA) of the data was carried out by SAS 9.1.3, SAS Inc. The difference

between treatments means was compared by Duncan's multiple range tests.

Results

Shoot growth parameters and physiological characteristics of almond genotypes were significantly affected by PEG treatments (Table 1). These results showed that there was a significant difference in all studied characteristics (Table 1).

Table 1. Analysis of variance results from the measurement of physiological and morphological characteristics of plantlet.

Sources of variation	df	Mean Square (MS)				
		Plantlet Height	Number of developed leaves	Proline	Ion leakage	RWC
Cultivar	4	42.39**	21.475**	230.33**	160.69 **	27.05 *
Treatment	3	1421.75**	239.128**	4155.18**	925.93 **	1045.17 **
Treatment *Cultivar	12	9.24*	3.419*	109.76**	12.11 *	11.21 n.s
Error	40	4.28	1.683	15.17	3.38	8.65
CV.	-	11.17	18.16	11.58	14.58	12.30

^{ns}: Non significant;*: Significant at the 5% level;** Significant at 1% level

Effect of osmotic stress on the number of developed leaves

According to the results (Table 1), there was a significant difference between control and stress treatments in the number of developed leaves. By increasing stress levels, the number of developed leaves was significantly reduced. Also, the number of leaves and the interaction between cultivars and treatments was significant at the 5% level, which showed different

reactions in different stress levels. The minimum number of developed leaves was recorded in 6% PEG treatment of 'Ferragnes', while 'Supernova' and 'Tuono' were less affected by stress, and different levels of stress kept their number of leaves higher than other cultivars (Fig.1).

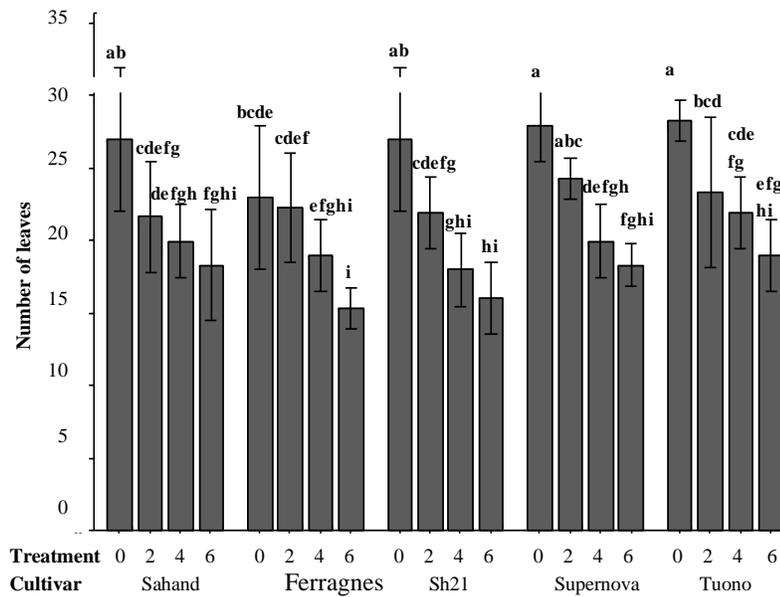


Fig.1. Effect of osmotic stress on number of developed leaves

Effect of osmotic stress on plantlet height

The results showed that an increase in osmotic stress causes a significant reduction in plantlet height. Least plantlet heights in all cultivars were recorded in 6% PEG treatment. Between all studied cultivars, the shortest

plantlet height was observed in ‘Ferragnes’ followed by ‘Shahroud 21’, while ‘Supernova’ and ‘Tuono’ were significantly less affected by stress (Fig.2).

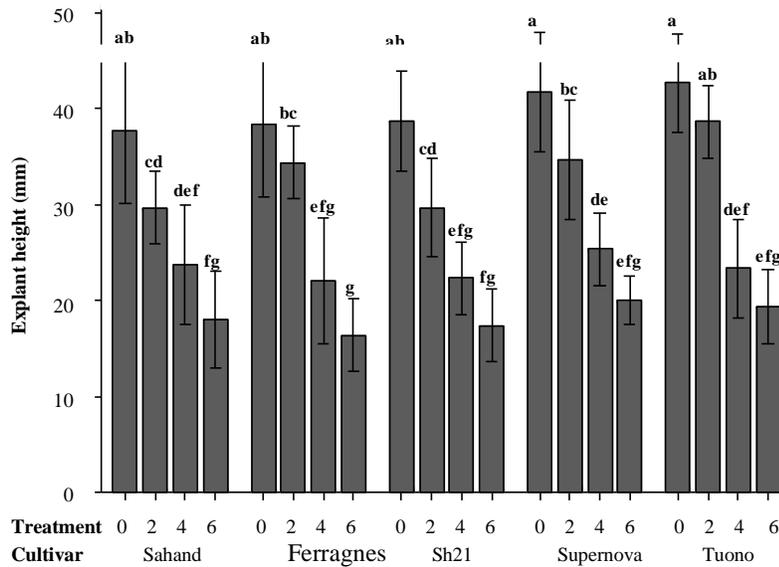


Fig. 2. Effect of osmotic stress on plantlet height.

Effect of drought stress on cell membrane stability

In all cultivars, ion leakage significantly increased as the drought stress level increased. Also, there was a significant difference in ion leakage between all treatments. As seen in the results, sensitive cultivars such as ‘Ferragnes’ and ‘Shahroud 21’ have more ion leakage at higher stress levels while resistant cultivars

like ‘Supernova’ and ‘Tuono’ experienced lower damage at their cell membrane and have significantly less ion leakage comparing to other studied cultivars. The ‘Sahand’ cultivar showed an intermediate performance comparing to others (Fig. 3).

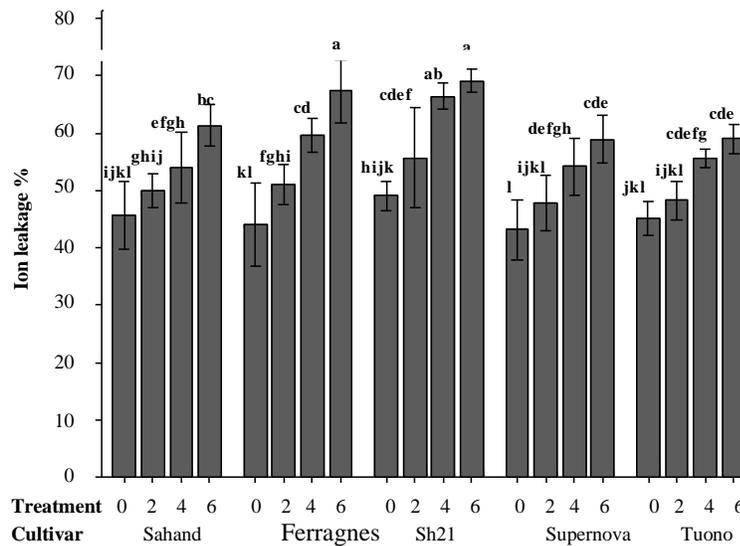


Fig. 3. Effect of osmotic stress on ion leakage of explants

Effect of osmotic stress on the relative water content (RWC) of leaves

The results showed that there was no significant difference between the cultivars in the level of RWC (Fig. 4). However, there was a significant difference between the treatments in regards to water content. As the osmotic stress increased, the relative water content of

leaves significantly decreased (Fig. 5). The lowest amount of RWC was recorded in 6% polyethylene glycol treatment of ‘Ferragnes’ while the highest amount of RWC in 4 and 6 percent of polyethylene glycol levels were recorded in ‘Supernova’.

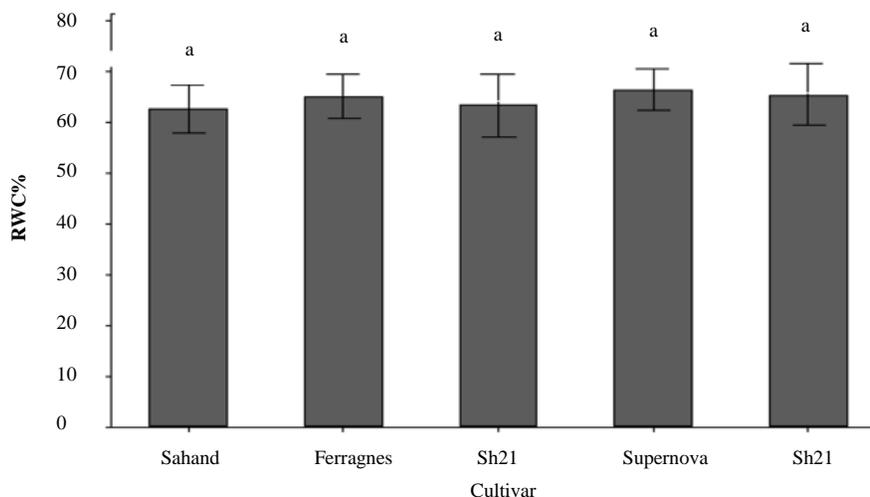


Fig. 4. Effect of cultivar on leaf relative water content of explants

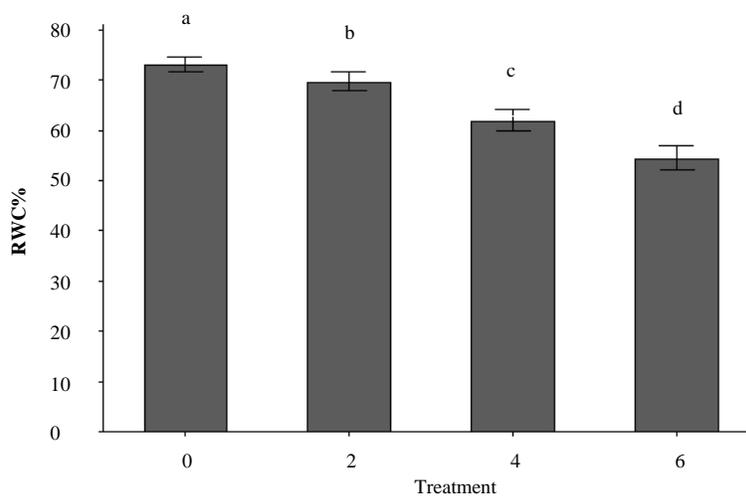


Fig. 5. Effect of osmotic stress on leaf relative water content of explants

Effect of osmotic stress on Proline

There was a significant difference in the content of proline between all treatments. The difference between all cultivars was significant as well (Table1). As seen in

Fig. 6, the highest amount of proline content was recorded in 6% polyethylene glycol treatment and in ‘Ferragnes’ followed by ‘Shahroud 21’ cultivars.

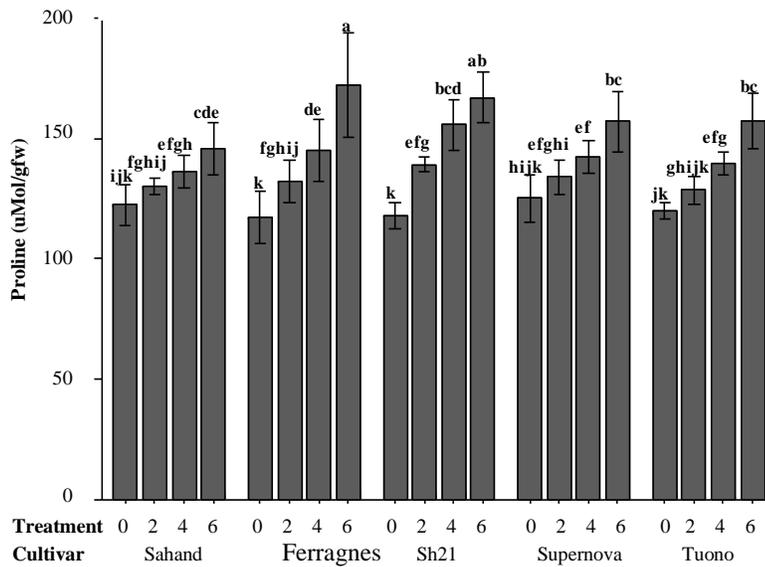


Fig. 6. effect of osmotic stress on explants' free proline content of leaves.

Discussion

This experiment evaluated the responses of different almond genotypes to PEG induced drought stress under in vitro conditions. The results showed that physiological and morphological characteristics of almond explants were significantly affected under drought stress. There were genetic differences between studied cultivars from the point of drought resistance. However, some cultivars were genetically more resistant to drought. Different levels of PEG in medium caused different responses in plantlets, as shown in the Figs. 7

and 8. Growth indicators were affected by intensity of treatments. There was a significant interaction between cultivars and treatments. According to these results, growth indicators were affected by the interaction between cultivars and treatments. Proline was significant at 1% level and other indicators such as ion leakage, number of developed leaves and plantlet height were significant at the 5% level. The RWC was not affected in the different treatment groups and cultivars (Table 1).



Fig. 7. Plantlets of 'Tuono' (Left: Control & right: 6% treatment).



Fig. 8. Plantlets of 'Shahroud 21' (Left: Control & right: 6% treatment).

A decrease of growth indicators in drought condition is known as a general response of plants to drought stress. Other research has shown that a long-period application of PEG in *in vitro* condition will cause a reduction in plant growth and recovery. Reductions of growth indicators of plantlets in osmotic stress condition may be due to the inability to absorb water. The reduction of cell turgescence potential in response to drought stress leading to a reduction in plant growth is a widely accepted fact. (Hsiao, 1973)

The results showed that under stressful conditions, the number of developed leaves was significantly reduced (Fig. 8). A reduction in the number of developed leaves is a defense mechanism against drought tolerance, which probably helps plant to absorb less light and therefore, has less transpiration surface. This issue is also mentioned in research carried out by Molassiotis *et al.* (2006) and Sivritepe *et al.* (2008). There was also a significant reduction in plantlets height under stressful conditions. Sorkheh *et al.* (2010) conducted research on eight species of almond using PEG and sorbitol as osmotic agents. Their results showed that there was no difference between all species in the control group. In the stress treatment groups, there was a significant difference between all cultivars. Also, in different treatment groups of a specific cultivar, plantlets of the control group have significantly higher

growth indicators than other treatments, which correlate to results of this study.

Based on the results of this study, ion leakage of plantlets was reduced under stressful conditions. Ion leakage of sensitive cultivars ('Ferragnes' and 'Shahroud 21') was higher than in other groups. Cell membrane is one of the main parts of a plant that is being affected during various kind of stresses. Ion leakage is a physiological index that is widely used to study a plant's resistance to drought and temperature stresses. Drought stress causes an increase in the leakage of materials from leaf cells. Ion leakage index is used to determine the severity of damage to the cell membrane and evaluation of plant's resistance to abiotic stresses such as drought, salinity and changes in temperature (Karimi *et al.*, 2012a). In similar results reported by Savee *et al.* (1990), ion leakage of leaf samples of kiwi fruit increased in response to drought stress. Sairam and Srivastava (2002) reported that genotypes, which are sensitive to salinity, showed a significant reduction in their cell membrane stability index. Based on the research results of Saneoka *et al.* (2004), plant cells that are resistant to osmotic changes during drought stress have more cell membrane stability. Karimi *et al.* (2012) showed that *in vitro* drought stress significantly increased the ion leakage of common fig's leaf samples, and a plant's drought resistance has an

inverse relationship with leaf samples ion leakage under drought stress condition.

The results of RWC content of plantlet leaves showed that studied cultivars have a significant difference in their ability to keep their water content level and when the osmotic stress become more severe, resistant cultivars like 'Supernova' and 'Tuono' lose their RWC with lower ratio. In other studied cultivars, such as 'Ferragnes' and 'Shahroud 21,' the decrease of RWC is higher. Hence, genetic differences between cultivars of almond can have a direct effect on cultivars resistance to drought stress. Wang (2014) studied rubber trees and measured relative water content of leaves of seedlings under drought stress. The results were similar to the results of this study. Under severe drought stress, relative water content of the samples will be reduced to about 20%. Also In a study on oak seedlings under drought stress (Valero-Galvan *et al.*, 2013) showed that by increasing the stress level, the relative water content of the sample was reduced.

In this study, more proline content was recorded in the sensitive cultivars. As the drought stress increased in the treatment groups, in resistant cultivars such as 'Supernova' and 'Tuono', proline accumulated with a lower ratio. This proves that in long periods of drought stresses and in humid conditions, high levels of proline content in almond leaves cannot aid in resisting drought stress. In the first stages of drought stress in almond cultivars, other osmotic agents play a role in osmotic regulation of leaves. It can be concluded that in short periods of drought stresses, proline accumulation is a reliable indicator that the plant is entering drought stress. On the other hand, during long periods of drought, plants that have relatively higher amount of proline content are able to better tolerate the stressful conditions. Trotel *et al.* (1996) reported that proline accumulation in plants that are under salinity stress is not a fast reaction, and this high level of proline content stays in plant's tissues for a long time.

Conclusions

The results showed that drought stress increased ion leakage and proline content, while it reduced the RWC, plantlet height and number of developed leaves. Based on these results, it can be concluded that the 'Supernova' and 'Tuono' were less stressed compared to other cultivars, while 'Shahroud 21' and 'Ferragnes' were more affected than others. 'Sahand' also showed an intermediate performance compared to other cultivars.

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