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Responses of Almond Genotypes to Osmotic Stress Induced In Vitro

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Abstract

Drought is one of the major limitations to crop production worldwide. This study was conducted to evaluate the response of five almond genotypes and peach×almond hybrid GF677 to drought stress *in vitro*, and screening drought tolerance. Explants subjected to polyethylene glycol osmotic stress (0, 3.5, and 7.0% WV) on the MS medium. Increasing PEG level in the medium significantly reduced fresh weight and leaf growth indices of the explants. Concentrations of chlorophylls, anthocyanins and carotenoids were significantly reduced under osmotic stress. Drought sensitive genotypes 'B-124', 'Sepid', 'Mamaei' and 'Ferragnès' showed stunted growth with high rate of leaf abscission under osmotic stress. Under osmotic stress, leaf water content, cellular membrane stability and pigments concentration were significantly higher in the leaves of tolerant genotypes 'Supernova' and GF677. The results revealed that carotenoids and anthocyanins may be involved in protecting almonds against drought stress. Based on their responses to the osmotic treatments, almond genotypes were divided into drought tolerant ('Supernova' and GF677), semi-sensitive ('B-124' and 'Sepid') and drought sensitive ('Mamaei' and 'Ferragnès').

Keywords: Almond, Anthocyanins, Carotenoids, Leaf pigments, Osmotic stress, Polyethylene glycol.

Introduction

Almond (*Prunus dulcis*) is a major nut crop cultivated around the word. Almond originated from central and southwest Asia, and represents divergent evolution under cold and xerophytic environments (Shiran *et al.,* 2007). Almond can control water loss and shows xeromorphic responses to drought stress condition and generally known as a drought tolerant plant (Alarcon *et al.*, 2002). However its production is very susceptible to drought stress. Studies have shown that almond productions can be reduced to about 50% under drought conditions (Gomes-Laranjo *et al.*, 2006). Isaakidis *et al.* (2004) and Barzegar *et al.* (2012) reported high rate of leaf shedding and reduced stomatal conductance and carbohydrates metabolism of almond under drought stress. Rouhi *et al.* (2007) and Yadollahi *et al.* (2011) stated that reduced leaf water content and leaf water potential under reduced soil water availability, adversely affect growth of almonds.

Reduced water activity in the plant tissues under drought stress induces reactive oxygen species accumulation by altering cells metabolism, which leads to oxidative stress and structural damages (Sircelj *et al.*, 2007). Cell membrane lipids and chloroplasts are greatly susceptible to the drought stress induced oxidative stress. Cell membrane injuries due to peroxidation of lipids under drought stress leads to loosing of cell membrane integrity and increased electrolyte leakage from cells (Liang *et al.*, 2003). Egert and Tevini (2002) stated that reduced chlorophylls content in the leaves is one of the primitive signs of oxidative stress in the leaves. Rouhi *et al.* (2007) and Yadollahi *et al.* (2012) reported reduced cell membrane stability

and increased chlorophyll degradation in the leaves of drought sensitive almonds under severe water stress. Yadollahi *et al.* (2012) and Barzegar *et al.* (2012) concluded that structural damages to photosynthesis apparatus and reduced efficiency of photosystem II probably are involved in reduced photosynthesis rate of almonds under prolonged severe stress.

A great diversity in drought tolerance of almonds has been reported by Rouhi *et al.* (2007), Yadollahi *et al.* (2011) and Barzegar *et al.* (2012). Screening and utilizing drought tolerant almonds is a sustainable approach to cope with drought stress in semi-arid and arid conditions. However, screening of drought tolerant genotypes of woody plants such as almond is a time consuming and costly process. Under such circumstance, *in vitro* studies have been introduced as a reliable alternative screening method to the field experiments (Karimi *et al.*, 2012). Save and Adillon (1990), Aazami *et al.* (2010) and Wani *et al.* (2010) reported successful screening of drought tolerant woody plant species using *in vitro* studies.

This study was aimed to evaluate five high yield and late bloom almond genotypes to drought stress induced *in vitro*. Physiological responses of the almond genotypes were compared to the drought tolerant peach×almond hybrid GF677 as a benchmark in order to screen drought tolerance. Drought tolerant genotypes were introduced based on the results.

Materials and Methods

Current season shoots of three Iranian almonds ('Mamaei', 'Sepid', and 'B-124') and two foreign almond cultivars ('Supernova' and 'Ferragnès'), and also peach×almond hybrid GF677 were excised from 4-year-old trees of the Almond

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Research Center, Karaj, Iran. For sterilization, shoots were placed under running tap water for an hour and submerged in 3% mercury chloride solution for 90 seconds. Shoots were rinsed three times in sterile distilled water and then explants with 15–20 mm length (single node) were prepared.

Explants were individually transferred to jars containing 15 ml of the Murashige and Skoog (MS) basal medium. The medium were supplemented with 30 g L^{-1} sucrose, 1 g L^{-1} benzyl adenine (BA) and 8 g L^{-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 autoclaving at 121˚C for 15 min. Cultures were maintained at 25.0±3˚C and 16:8 h photoperiod of cool-white light at 1250 lux. After 30 days, uniform developed explants were excised and transferred to the same medium but containing 0.1 mg L^{-1} BAP. The explants were maintained at the same conditions described above for 30 days.

Uniform developed explants were selected and transferred to the MS media containing different concentrations of poly ethylene glycol 6000 (PEG) namely 0, 3.5%, and 7%. No plant growth regulator was added to these media. The incubation conditions were the same as described above.

After 40 days, at the end of experimental period, fresh weight, leaf number, total leaf area, and mean leaf area of 5 top developed leaves were recorded. Leaf water content was measured for the first three fully expanded leaves at the top of each explant. Leaves were dried at approximately 70˚C for 48 h to determine dry mass. Leaf water content was calculated using the following equation (Griffiths *et al.*, 2006):

Leaf water content = (leaf fresh weight - leaf dry weight) / leaf dry weight

Where SW, FW and DW were the saturated weight, fresh weight and dry weight of leaves, respectively.

Electrolyte leakage in the leaf tissue was measured by using the method described by Blum and Ebercon (1981). Photosynthesis pigments were measured in leaf discs with a known area (10×50.24 mm^2 discs). The discs were cut into smaller pieces and extracted with 5 mL of DMSO at 70 ^oC for 30 min (Hiscox and Israestam, 1978). Absorbance of the extract was measured by a spectrophotometer at 470, 646 and 663 nm. Total chlorophyll content and the ratio of concentrations of chlorophylls to carotenoids ratio were determined following the equations proposed by Wellburn (1994).

Five hundred mg leaf material was homogenized in 1 ml of acidified (1% HCl) methanol and maintained at 4 ˚C for 24 h. The absorption of anthocyanins at 550 nm was measured by a spectrophotometer. Concentration of anthocyanins was determined by using the extinction coefficient (Wagner, 1979):

ε550= 33,000 (cm^2/moll.)

The experiment was carried out as a factorial experiment based on a completely randomized design (CRD) with two factors and 5 replications per treatment and two jars per replication. The first factor was the different concentrations of PEG (0, 3, and 6%), and the second was the different almond genotypes. Statistical analysis of the data was carried out by SPSS 16.0 (SPSS Inc.). The results subjected to an analysis of variance (ANOVA) and difference among treatments means were compared by using Duncan's multiple range test at $P \leq 0.05$.

Results

Increasing PEG level in medium significantly reduced fresh weight of the explants (Table 1 and Fig.1). The lowest fresh weight obtained in 'Ferragnès' explants on media containing 7% PEG. Leaf growth indices involving leaf number, mean leaf area, and total leaf area of the explants were significantly reduced by increasing PEG level in the media (Table 1). Leaf growth indices of different almond genotypes were significantly different (Table 1). The highest number of leaves was found in control treatment and 'Mamaei' had the highest number of leaves in control media. On the other hand, number of necrosed leaves was significantly increased by increasing PEG concentration in the media. The highest number of necrosed/fallen leaves, 8.8 leaves per explant, was found in 'Mamaei' on media containing 7% PEG. 'Supernova' and GF677 had the lowest number of necrosed/fallen leaves (3.5 and 3.6 leaf per explant, respectively) (Fig. 1). Mean leaf area of 'Supernova' on control media (1.7 cm^{-2}) was significantly higher than the other genotypes. Increasing PEG concentration in the media significantly reduced leaf area of the explants and the lowest leaf area was found in 'Sepid' and 'Mamaei' on 7% PEG containing media (0.9 and 1.0 cm^{-2}). Explants of 'Supernova' had the highest leaf area under osmotic stress (Fig. 1).

Table 1. Results of analysis of variance (mean squares) for effect of osmotic stress and almond (*Prunus dulcis*) genotypes on leaf growth indices of *in vitro* explants.

Source of Variations	*df*	Explant FW	Leaf No.	Necrosed/Fallen Leaves	Mean Leaf Area
Genotype	5	0.045**	45.16^{ns}	31.09**	0.54**
Drought Stress	2	0.118**	214.57**	289.61**	0.99**
Genotype × Drought Stress	10	0.005^{ns}	33.96^{ns}	8.61*	0.02^{ns}
Error	72	0.008	22.63	4.09	0.06

df: degree of freedom; *, ** significant at 5% and 1% probability levels, respectively; ns: non-significant

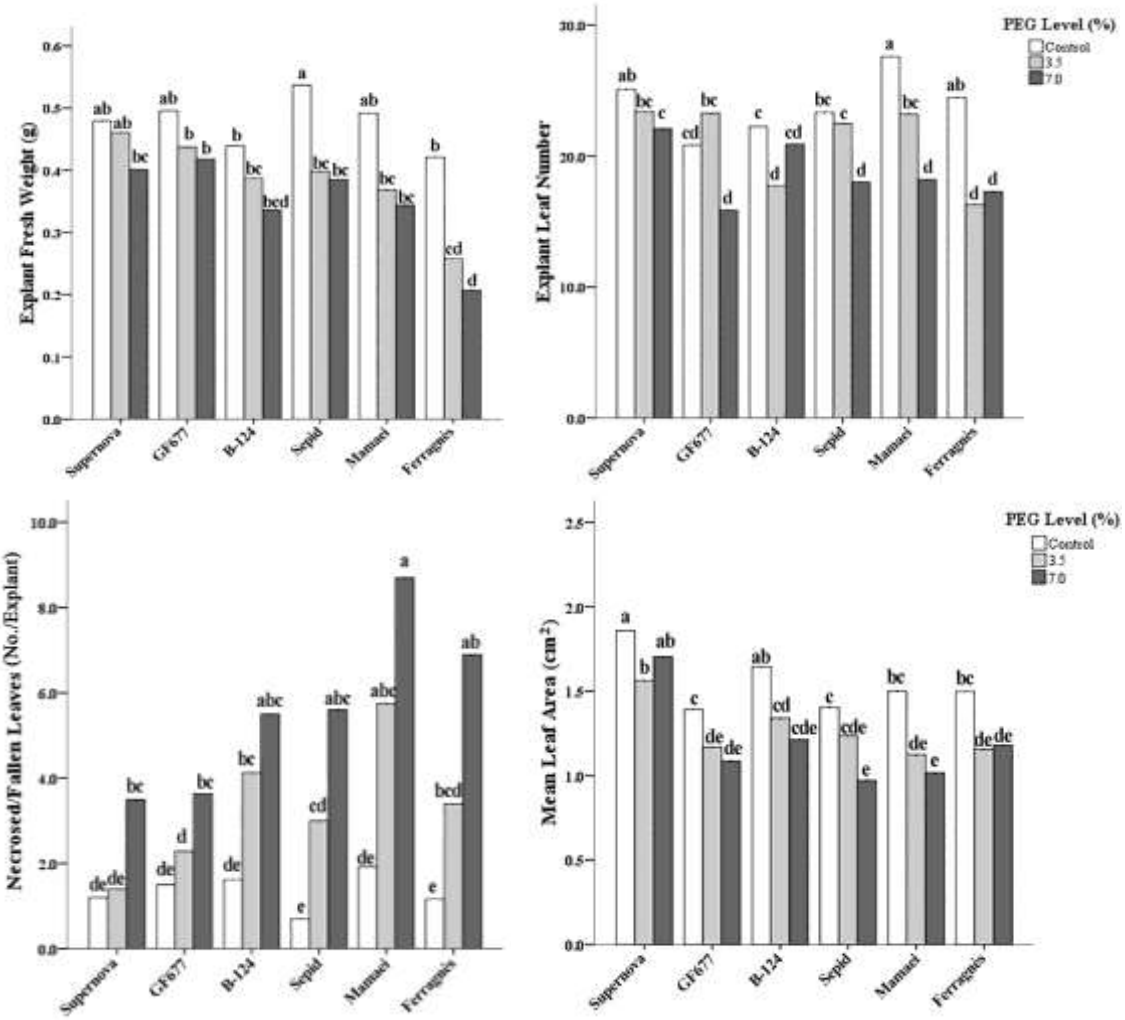

Fig. 1. Effect of osmotic stress on growth parameters of almond genotypes explants.

Osmotic stress significantly affected leaf water content (LWC) of the explants (Table 2), and the lowest LWC was obtained in 'B-124' (67%) and 'Mamaei' (69%) on media containing 7% PEG. However, osmotic stress did not affect LWC of 'Supernova' explants (Fig. 2). Increasing PEG level in media significantly increased electrolyte leakage (EL) in the leaves of the explants. (Table 3). EL was significantly higher in 'Sepid' (43%), 'Mamaei' (44%) and 'Ferragnès' (46%) on media containing 7% PEG (Fig. 2).

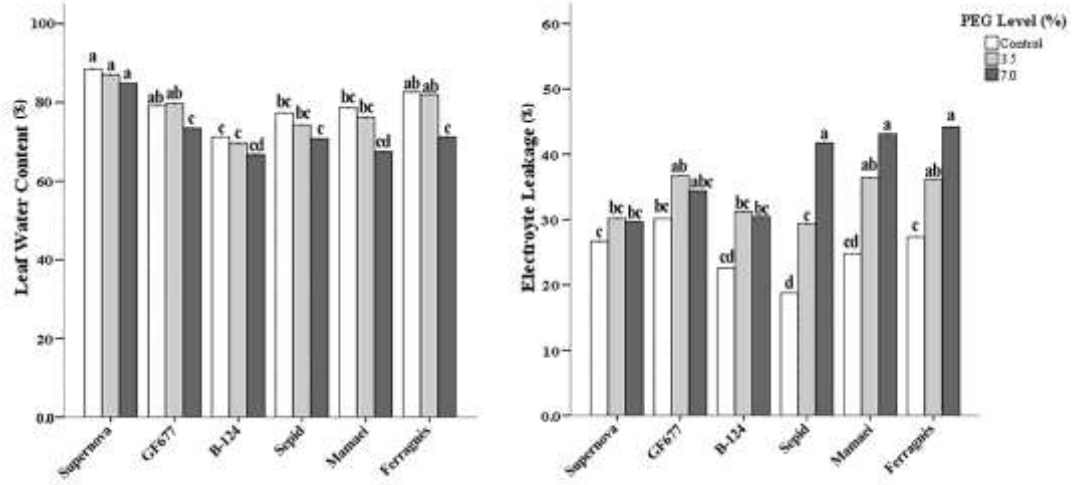

Fig. 2. Effect of osmotic stress on electrolyte leakage and leaf water content of almond genotypes.

Table 2. Results of analysis of variance (mean squares) for effect of osmotic stress and almond (*Prunus dulcis*) genotypes on leaf water content (LWC), electrolyte leakage (EL), and concentration of leaf pigments.

Source of Variations	*df*	LWC	EL	Total Chl.	Chls : Carotenoids	Anthocyanins
Genotype	5	0.036**	0.013^{ns}	3009.51**	0.79**	0.24**
Drought Stress	2	0.030**	0.100**	33490.88**	8.96**	0.22**
Genotype × Drought Stress	10	0.001^{ns}	0.008^{ns}	481.92^{ns}	0.098^{ns}	0.04^{ns}
Error	72	0.007	0.006	857.81	0.23	0.01

df: degree of freedom; *,** significant at 0.05 and 0.01 probability levels, respectively; ns: non-significant

The effects of experimental treatments on concentration of total chlorophylls in the leaves of almond explants shows in Table 2. Photosynthesis pigments significantly reduced under osmotic stress (Fig. 3). The lowest chlorophylls content was found in the leaves of 'B-124', 'Sepid', 'Mamaei' and 'Ferragnès' on media containing PEG 7%. The ratio of chlorophylls to carotenoids showed significant reduction under osmotic stress. 'B-124', 'Sepid', 'Mamaei' and 'Ferragnès' had the lowest chlorophylls to carotenoids ratios under PEG 7% treatment (Table 2 and Fig. 3).

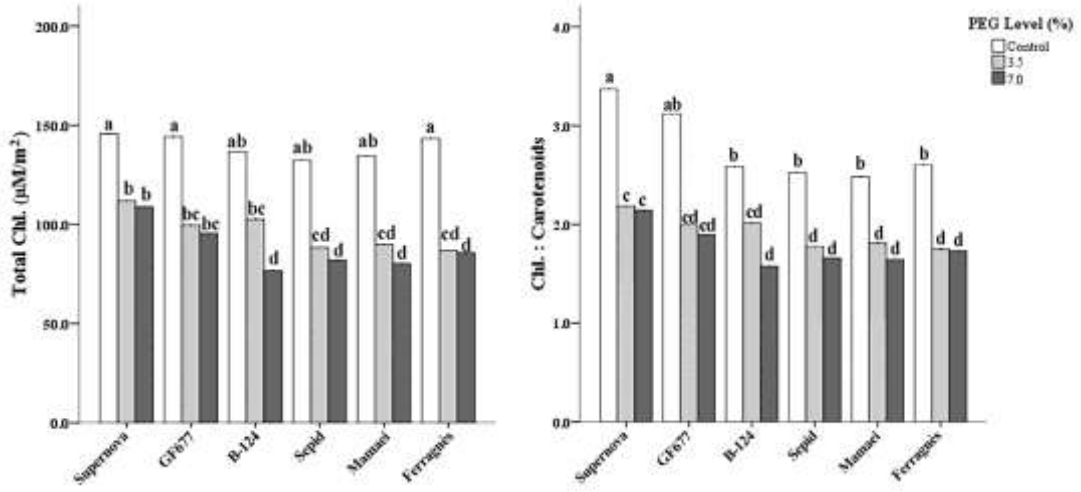

Fig. 3. Effect of osmotic stress on concentration of photosynthesis pigments and chlorophyll to carotenoid ratio in the leaves of almond genotypes.

Concentration of anthocyanins in the leaves of almond genotypes was significantly reduced by increasing PEG level in the media. The lowest anthocyanins concentration obtained in the leaves of 'Ferragnès' under 3.5 and 7.0% PEG treatments (4.6 and 4.7 µmol g FW^{-1}, respectively) (Fig. 4).

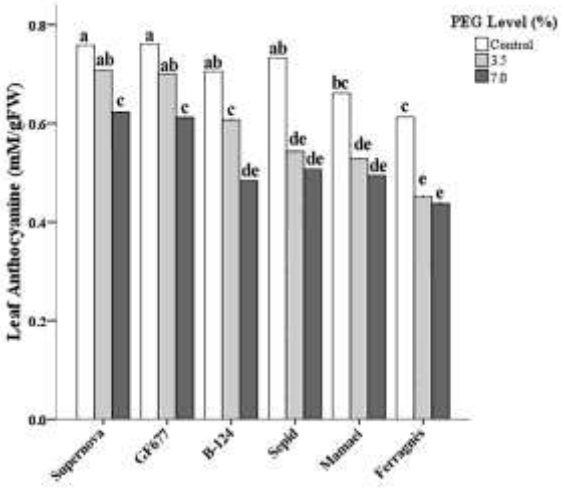

Fig. 4. Effect of osmotic stress on concentration of anthocyanins in the leaves of almond genotypes.

Discussion

Limitation of shoots or leaves growth indices is a general response to water limitation. Other researchers have also reported the reduction of growth and regeneration of explants under prolonged osmotic stress induced *in vitro* (Dami and Hughes, 1995; Al-Khayri and Al-Bahrany 2004). Growth limitation is mainly due to loss of turgor pressure which reduces cell elongation (Syversten, 1985). Salisbury and Ross (1992) stated that cell growth is the most sensitive process to drought stress. As leaf water content (LWC), data show the reduction of water content of the almond explants during osmotic stress may impose limitation on explants elongation and leaf expansion. Researches on apple (Molassiotis *et al.* 2006) and cherry (Sivritepe *et al.*, 2008) have pointed out that reduction in water content is the reason for growth limitation under PEG induced osmotic stress *in vitro*.

Leaf area development is very sensitive to osmotic stress. Changes in water and mineral absorption may trigger limitation of leaf expansion under drought conditions (Marron *et al.* 2003). Hsiao (1973) stated that reduction in leaf area development reduces light absorption surface which constrains photosynthesis and leads to plant growth under drought conditions. However, under *in vitro* conditions it seems not to be true owing the sugar source used in the media which can be consumed for growth process by the explants. In this study, reduced water absorption probably is the main reason for growth reduction of almond explants.

Explants showed reduced number of leaves in the PEG treatments because of limitation of new leaves regeneration and increase in defoliation of explants. Brito *et al.* (2003) also reported defoliation of olive explants under *in vitro* drought stress. Losing leaves under osmotic stress is one of the main reasons for reduced fresh weight of the explants.

Elevated EL in the leaves of almonds was in coincidence with cell dehydration. Saneoka *et al.* (2004), Sivritepe *et al.* (2008), and Karimi *et al.* (2012) also reported drought stress damages the cell structures. Malfunctions of cell metabolism during dehydration leads to reactive oxygen species (ROS) formation and damage to cell membrane which results in EL increase. Leaf necrosis, decrease in chlorophyll concentration and yellowing of leaves may be referred to visual symptoms of extreme cellular damages under severe drought stress. Structural damages to chloroplasts due to ROS formation and/or photo degradation of the pigments probably led to loss of chlorophylls under drought stress (Anjum *et al.*, 2011). Chlorophylls concentration was remained higher in the leaves of 'Supernova' and GF677. Chlorophyll maintenance under drought stress in tolerant genotypes has also been reported by Kraus *et al.* (1995) and Sairam *et al.* (1998).

Our data revealed that concentration of leaf carotenoids remains higher than chlorophylls during water stress. Carotenoids as photoperotective compounds have critical role in limiting structural damages to chloroplasts and chlorophylls by quenching triplet chlorophyll. Carotenoids are also responsible for scavenging of singlet oxygen (Nishida *et al.*, 2007), thus preserving carotenoids in the leaves may be led to less structural damage and higher chlorophyll concentration during water stress. The higher ratio of chlorophyll to carotenoids in the leaves of 'Supernova' and GF677 indicates the capacity of higher carotenoid concentration to protect the photosynthetic apparatus (Loggini *et al.*, 1999).

Anthocyanins concentration was significantly reduced under drought stress. However, it remained higher in the leaves of the drought tolerant genotypes, 'Supernova' and GF677. Complementary to their photoprotective function, anthocyanins have also demonstrated potent antioxidant capabilities (Edreva *et al.*, 2008). Close and Beadle (2003) stated that anthocyanins could provide widespread cellular protection for cellular membranes, organelles, and DNA. Preserving anthocyanins at higher level in the leaves of 'Supernova' and GF677 may be considered as another protective mechanism against drought stress.

Conclusion

In this study, the responses of almond genotypes were investigated to *in vitro* osmotic stress. The data suggested the possibility of screening drought tolerant almond genotypes under *in vitro* condition. Water saturation deficit and cellular membrane stability were found good parameters to screen drought tolerance in almond. Anthocyanins and carotenoids were suggested to be involved in drought tolerance of almonds.

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