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Original Research Article

In vitro Screening of Almond (*Prunus dulcis* (Mill.)) Genotypes for Drought Tolerance

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ABSTRACT

Screening and identifying drought tolerant almond genotypes is required in order to improve and stabilize almond production under semi-arid and arid conditions. In this study, we evaluated responses of five high yield and late blooming almond genotypes to drought stress induced *in vitro*, and compared their drought tolerance to the drought tolerant peach×almond hybrid 'GF677'. Explants established on the MS medium and subjected to three polyethylene glycol (PEG) osmotic stress for 40 days. Plant growth indices, leaves relative water content (RWC), membrane stability index (MSI), were significantly reduced under drought stress. On the other hand, proline concentration and number of lateral shoots were significantly increased under drought stress. Drought tolerant genotypes maintained higher levels of RWC, and MSI under drought stress. Growth of drought tolerant cultivars was more stable. Proline accumulation in the explants was found to be a general response of almond to drought stress. The results suggested that drought tolerant almond genotypes may be screened by *in vitro* experiments.

Key words: Almond, Drought stress, Leaf pigments, Polyethylene Glycol, Proline.

Abbreviations: Almond, Leaf Relative Water Content (RWC), Membrane Stability Index (MSI), Proline, Polyethylene Glycol (PEG).

INTRODUCTION

Climate change has caused a rise in temperature, less precipitation, increasing variability in rainfall and reducing recharge of underground aquifers in many areas (Pray et al. 2011). It is predicted that global warming will cause a massive drought and take over half the land surface on our planet in the next 100 years. Severe drought makes modern agriculture virtually impossible. Using drought tolerant cultivars is the most sustainable approach to reduce the pressure of the periodic drought.

Almond (*Prunus dulcis* (Mill.) D.A. Webb) generally known as a drought tolerant plant; however, drought conditions limit quality and quantity of its production (Gomes-Laranjo *et al*. 2006, Camposeo et al. 2011). Hutmacher et al. (1994) and Nanos et al. (2002) pointed out that irrigation was the most important factor, determines almond yield. Gomes-Laranjo et al. (2006) reported that reduced water potential under drought conditions resulted in growth limitation, massive leaf abscission, and reduction in kernel weight of almonds.

The aim of the current study was to evaluate the response of five late bloom and high yield almond genotypes to drought stress and compare their responses to the drought tolerant peach almond hybrid, GF677 rootstock, under *in vitro* conditions. The effects of induced drought stress *in vitro* have been reported on many crops, including kiwi fruit (Save and Adillon 1990), alfalfa (Dragiiska et al., 1996), olive (Brito et al. 2003), sunflower (Turhan and Baser 2004), tomato (Aazami et al. 2010), rice (Wani et al. 2010), and common fig (Karimi et al. 2012). Researchers introduced such techniques as a useful tool to screen different genotypes, based on their responses to induced drought stress *in vitro* (Save and Adillon 1990, Aazami et al. 2010, Wani et al. 2010).

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 Almond 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 s. Shoots were rinsed three times in sterile distilled water and then explants with 15–20 mm length (single node) were prepared and individually transferred

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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℃ for 15 min. 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 but containing 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, 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, height, diameter, dry weight, shoot number, and specific leaf area (SLA) of the explants were measured. SLA of ten 10 mm diameter leaf discs was measured using the following formula:

$$SLA = 100 \times \text{Leaf disc area} / \text{Leaf discs dry weight}$$

Relative water content of explants' leaves (RWC) was measured by using ten 7 mm diameter leaf discs. The leaf discs of each treatment were weighed (FW). They were then hydrated until saturation (constant weight) for 48 h at 5 °C in darkness (TW). Leaf discs were dried in an oven at 105 °C for 24 h (DW). Relative water content was calculated according to the following expression (Filella et al. 1998):

$$RWC\% = FW - DW / TW - DW \times 100$$

Membrane stability index (MSI) was measured by using the method described by Blum and Ebercon (1981). Proline content was measured in 300 mg of leaf material via the method described by Bates et al. (1973). The absorbance was measured at 520 nm with a spectrophometer. L-Proline (SIGMA^{TM}) was used as standard.

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, 3.5, and 7%), and the second was the different almond genotypes. Analysis of variance (ANOVA) of the data was carried out by SPSS 16.0, SPSS Inc. The difference among treatments means were compared by using Duncan's multiple range test at $P \leq 0.05$.

RESULTS

Shoot growth parameters of almond genotypes were significantly affected by PEG treatments (Table 1). Dry weight, height, and diameter of explants were significantly reduced under drought stress (Fig. 1). Diameters of 'Supernova' and 'GF677' explants were significantly higher than the other cultivars.

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

Source of Variations	*df*	Explant dry weight	Height growth	Diameter	Shoot number
Genotype	5	0.012**	0.15 **	1.18**	2.05**
Drought Stress	2	0.063**	0.13*	1.73**	3.87**
Genotype × Drought Stress	10	0.001^{ns}	0.02^{ns}	0.12^{ns}	0.78^{ns}
Error	72	0.005	0.04	0.21	0.43

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

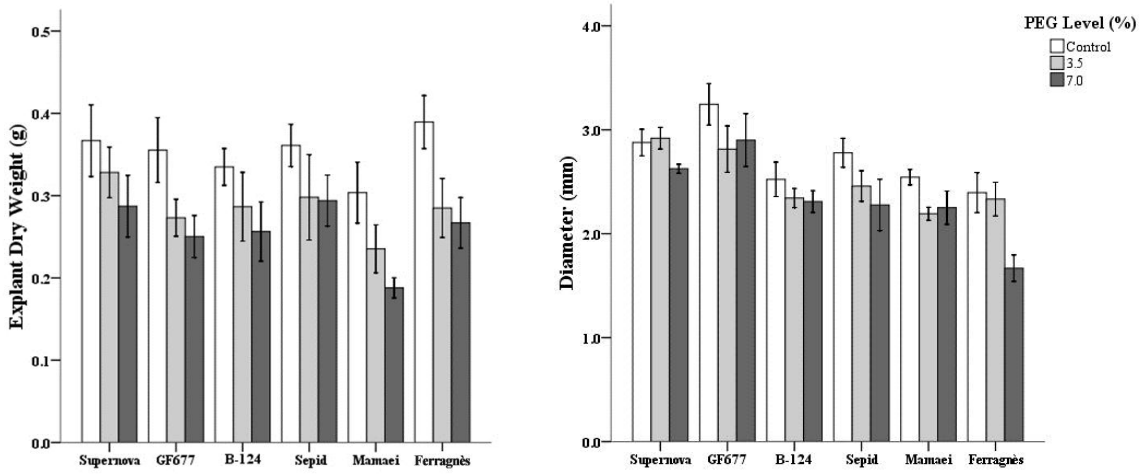

Figure 1. Effect of PEG induced drought stress on dry weight and diameter of almond genotypes explants.

Height growth of the explants was significantly lower on the media containing PEG. The lowest shoot growth was obtained on media containing 7% PEG. The inhibitory effect of PEG was significantly higher on 'Sepid', 'Mamaei, and 'Ferragnès'. PEG treatments significantly increased shoot regeneration, and shoot number was significantly higher in 'Mamaei' and 'B-124' explants (Fig. 2).

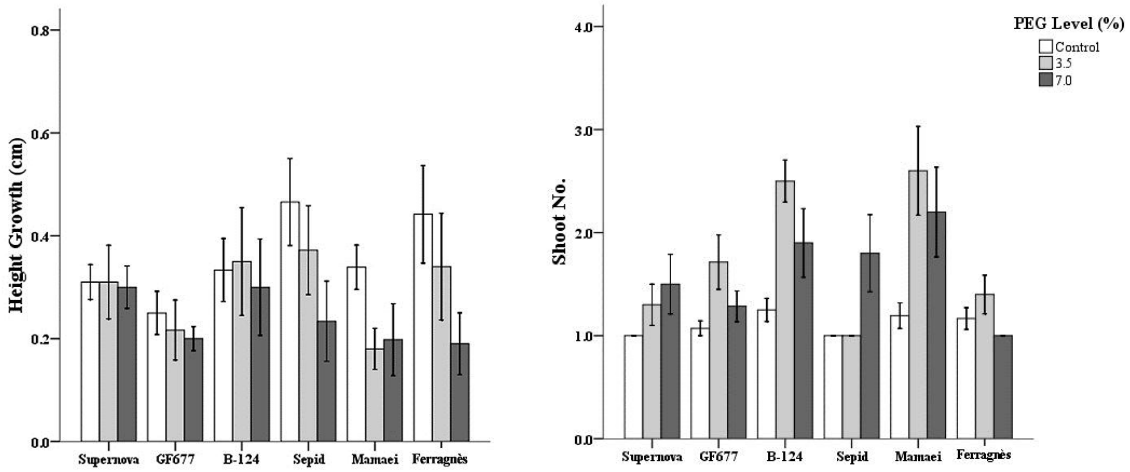

Figure 2. Effect of PEG induced drought stress on height growth and shoot number of almond genotypes explants.

Drought stress significantly affected SLA of the almond explants (Table 2). The lowest SLA values were found on media containing 7% PEG. The highest SLA values were found in 'Supernova', and the lowest value observed in 'Ferragnès' (Fig. 3). Relative water content of the explants' leaves was significantly reduced by increasing PEG level in the media (Table 2 and Fig. 4). Membrane stability index was significantly reduced in the presence of PEG in the media (Table 2 and Fig. 5). The lowest MSI values found in 'Ferragnès', 'Mamaei', and 'Sepid'.

Table 2. Results of analysis of variance (mean squares) for effect of drought stress and almond (*Prunus dulcis*) genotypes on specific leaf area (SLA), leaf relative water content (RWC), membrane stability index (MSI), and proline concentration in the explants.

Source of Variations	*df*	SLA	Leaf RWC	MSI	Proline
Genotype	5	40295.09^{ns}	119.64 ^{ns}	131.12 ^{ns}	2985.58*
Drought Stress	2	126959.79**	1068.66**	952.82**	15117.12**
Genotype × Drought Stress	10	13172.85^{ns}	70.22^{ns}	75.44^{ns}	498.81^{ns}
Error	72	17653.43	289.55	57.51	1152.45

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

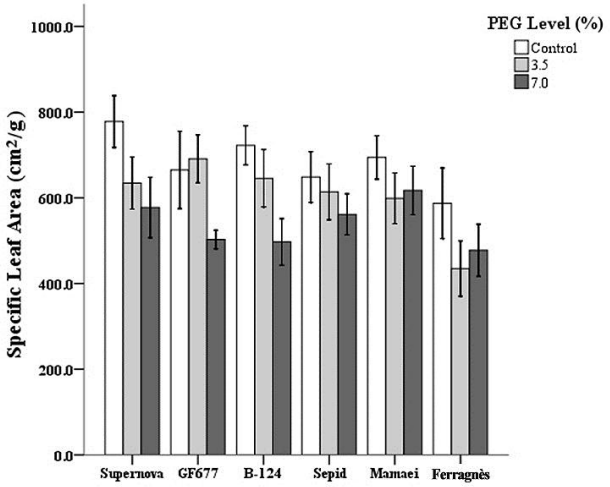

Figure 3. Effect of PEG induced drought stress on specific leaf area of almond genotypes explants.

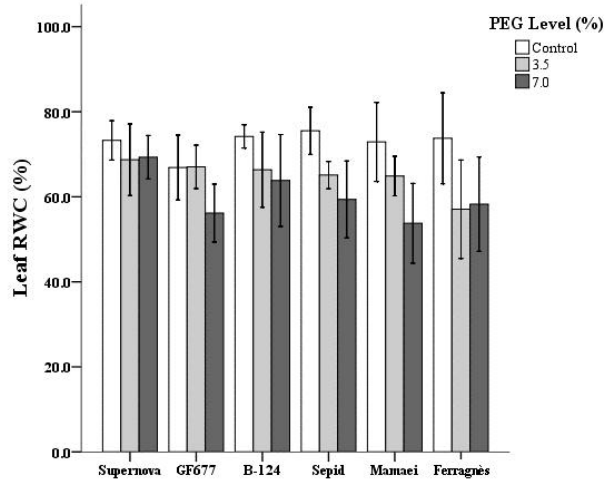

Figure 4. Effect of PEG induced drought stress on leaf RWC of almond genotypes explants.

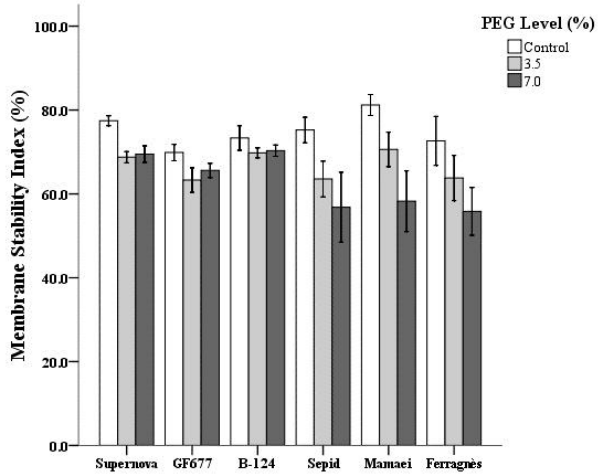

Figure 5. Effect of PEG induced drought stress on MSI of almond genotypes explants.

Increasing PEG level in the media significantly increased proline concentration in the explants. As it can be seen, concentration of proline in almond explants also was significantly different (Table 2). Proline concentration was significantly higher in 'Mamaei' and 'Ferragnès' (Fig. 6).

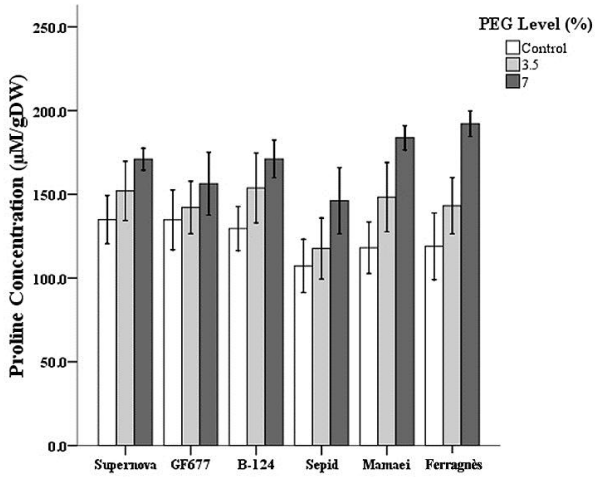

Figure 6. Effect of PEG induced drought stress on proline concentration in the leaves of almond genotypes explants.

DISCUSSION

In the current study, the responses of different almond genotypes to PEG induced drought stress under *in vitro* conditions has been evaluated. We grouped the almond genotypes into three categories: 'Supernova' and 'GF677' were classified as drought tolerant, 'B-124' and 'Sepid' was classified as semi-sensitive, and 'Mamaei' and 'Ferragnès' were classified as sensitive. Whit the exception of lateral shoot regeneration, growth indices of the almond explants were significantly reduced under drought stress. Explants of sensitive genotypes, 'Ferragnès', 'D-124', and 'Mamaei', produced more lateral shoots under drought stress treatments. However, these lateral shoots were weak and had limited growth. Stimulation of lateral shoot regeneration of the drought sensitive genotypes under drought conditions was probably due to growth inhibition of shoot apical meristem, or necrosis and decay of shoot apical meristem. The rate of survival for all genotype was 100%, although explants of sensitive cultivars showed some signs of die back i.e. severe defoliation and necrosis of shoot apical meristem. Limitation of growth indices is a general response to drought stress. Other researchers have also

reported the reduction of growth and regeneration of *in vitro* explants under prolonged *in vitro* drought treatments (Dami and Hughes 1995, Al-Khayri and Al-Bahrany 2004). Growth limitation is mainly due to loss of turgor pressure which limits the elongation of cells (Syversten 1985). Salisbury and Ross (1992) stated that cell growth is the most sensitive process to drought stress. As RWC data show, the reduction of water content of the almond explants during drought stress may impose limitation on explants elongation and dry matter accumulation. Researches on apple (Molassiotis et al. 2006) and cherry (Sivritepe et al. 2008) have also pointed out that reduction in water content is the reason for growth limitation under PEG induced drought stress *in vitro*.

Hasio (1973) stated that reduction in leaf area development under drought conditions reduces the light absorption surface which constrains photosynthesis and plant growth. However, under *in vitro* conditions it seems not to be true owing to lots of sugar used in the media which can be consumed for growth process by the explants. Moreover, as previous researches have mentioned, reduction in water content probably is the main reason for growth reduction of almond explants. Losing leaves under drought stress is one of the main reasons for reduction of explants dry weight (data not shown).

Previous researches also have shown reduction in SLA under drought stress *in vitro* or *ex-vitro* experiments (Rieger et al. 2003, Karimi et al. 2012). SLA positively correlated with mean leaf area. Previous As Bloom and Pnoel (1990) stated, reduction in SLA under drought conditions shows that suppression of leaf area development is more sensitive to drought stress than dry weight accumulation in the leaves. Changes in water and mineral absorption under drought conditions may trigger SLA decrease (Marron et al. 2003). Liu and Stützel (2004) showed that SLA negatively correlated with water use efficiency. Abrams (1988) and Rieger et al. (2003) also found that SLA is generally lower in genotypes or species adapted to more xeric environments. Decline in SLA was a general response of almond genotypes to drought conditions. Our drought tolerant genotypes 'Supernova' and 'GF677' generally had higher SLA on non-stressed media; however, rate of reduction in SLA under drought stress was higher in drought tolerant genotypes. Hence, it can be concluded that SLA measurement may be used as a physiological marker to screen drought tolerant almond genotypes.

RWC is a valuable parameter to evaluate water content of plant tissues (Kramer and Boyer 1995). There was no significant difference between RWC of different cultivars; it shows drought tolerant almond genotypes can tolerate dehydration better than the sensitive cultivars. MSI decline of almond genotypes was in coincidence with decreased RWC and cell dehydration. Sivritepe et al. (2008), and Karimi et al. (2012) also reported MSI reduction under drought stress. RWC reduction and cell dehydration bring about some malfunctions of cell metabolism which lead to reactive oxygen species (ROS) formation. ROS damage cell membrane and other cell structures which result in MSI decline. Leaf necrosis, decrease in chlorophyll concentration and yellowing of almond genotypes' leaves may be referred to as visual symptoms of extreme cellular damages under severe drought stress. Although MSI data showed that drought stress causes structural damages to the leaves, MSI remains significantly higher in the leaves of drought tolerant almond genotypes. Hence it can be concluded drought tolerant almond genotypes can tolerate prolonged dehydration periods.

It has been suggested that proline acts as an osmoregulator, an osmo-protector or a regulator of the redox potential of cells under drought stress (Ozden et al. 2009). Based on such findings, some researchers believe that proline accumulation helps plant tolerate drought stress conditions. Reducing in RWC or structural damages may trigger proline accumulation in plant leaves (Taylor 1996). RWC and MSI data presented in this study suggest that proline accumulation in the leaves of almond genotypes under drought stress may not be simply correlated with RWC level or structural damage of membrane. Proline accumulation in the almond explants was related to both RWC and MSI changes under drought stress. We found more proline accumulates in the sensitive genotypes ('Mamaei' and 'Ferragnès'). Therefore, it can be considered as a physiological marker to evaluate the drought stress pressure on almonds, which may be used to screen drought tolerant almond genotypes. Proline data also suggest drought tolerant almonds may use other mechanisms apart from proline accumulation and osmotic regulation to cope with drought stress conditions.

CONCLUSIONS

Our results showed that it is possible to screen drought tolerant almond genotypes via *in vitro* methods. The drought-tolerant almond genotypes showed lesser reduction of growth characteristics and better stability under drought stress. Drought tolerant genotypes showed the ability of dehydration without being injured. Proline data showed that proline accumulation in the almond leaves is a general response to drought stress and its concentration probably is not related to drought tolerance of the plant. These results may be indicating that almond is sensitive to oxidative stress and structural damages of drought stress, although it may tolerated dehydration via osmoregulation mechanisms. Hence it can be concluded that drought tolerance of almond genotypes may be result of a combination of some physiological traits.

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