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In vitro evaluation of drought tolerance in two grape (*Vitis vinifera* L.) cultivars

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Abstract

Abiotic stresses pose a major threat to agriculture. Therefore, developing plants that are more tolerant of these stresses is very important for improving crop productivity. Grapevines (*Vitis vinifera* L.) is an important fruit crop cultivated in the world. An *in vitro* experiment was designed to study the response of ‘White Seedless’ and ‘Flame Seedless’ cultivars of *Vitis* to drought stress. Treatments included four concentrations of PEG 6000, i.e., 0, 0.5, 1, and 2% (w/v), which were equivalent to 0, -0.035, -0.07, and -0.14 times the water potential, respectively. The single-node explants of *Vitis* grown on MS medium, supplemented with growth regulators BA (2 mg/l), NAA (0.2 mg/l), sucrose (30 g/l), agar (7 gr), and activated charcoal (200 mg/l), were transferred to the same medium but with different concentrations of PEG for 30 days. The results showed that the Flame Seedless cultivar had better growth characters than the White Seedless cultivar on the average of PEG concentrations. Flame Seedless also managed drought stress in terms of shoot length, the number of leaves per shoot, dry weight, chlorophyll a, chlorophyll b, and soluble carbohydrates more efficiently than White Seedless, and produced a high percentage of callus (87.5%) at the 1% PEG stress level. Although the White Seedless cultivar was not more vigorous than Flame Seedless but showed significantly higher proline content, non-significant reduction in relative water content, and a slightly lower reduction in shoot length, and fresh weight at 2% PEG as compared to the control. It seems that both grapevine varieties succeeded in dealing with the PEG drought stress with their special mechanisms.

Keywords: Drought stress; *In vitro* culture; Polyethylene glycol; *Vitis vinifera* L.

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Introduction

Drought is the most important abiotic stress that limits the growth and production of crop plants (Levitt, 1980; Karimi *et al.* 2012). Therefore, breeding for the tolerant genotypes to drought stress can prevent higher yield losses imposed by this stress. The tissue culture technique may be used as an alternative technique to the traditional methods for developing stress-tolerant plants (Rai *et al.* 2011). These techniques are advantageous because they save time and space and researchers can control more efficiently the environmental factors and treatments (Hussain *et al.* 2012). The *in vitro* techniques have been used to select for drought tolerance (Heyser and Nabors 1981; Fallon and Phillips 1989; Turhan and Baser 2004; Wani *et al.* 2010; Karimi *et al.* 2012) and salinity tolerance (Heyser and Nabors 1981; Cano *et al.* 1998; Barakat and Abdel-Latif 1996; Debez *et al.* 2006) in crop plants. Besides salinity and drought, a few reports are also available on the effect of some other abiotic stresses such as UV (Levall and Bornman 19973), metal (Roy and Mandal 2005; Rout and Sahoo 2007), and frost (Dörffling *et al.* 1993) on plants under *in vitro* conditions.

Polyethylene glycol (PEG), mannitol, and sorbitol have been used to stimulate osmotic stress for *in vitro* selection (Darko *et al.* 2019), but PEG has been most frequently used to impose water-deficit stress in plants (Jacomini *et al.* 1988). The imposed plants are characterized by several compounds such as proline and antioxidant enzymes (Rai *et al.* 2011).

Iran is the origin of commercial grapevines (*Vitis vinifera* L.) and there are numerous varieties of Persian origin (Winkler *et al.* 1974). The growth and production of grapevines depend on rainfall and, therefore, may be limited by drought conditions. Thus, identifying drought-tolerant grapevine genotypes could be beneficial for drought stress conditions. This study was aimed to evaluate the drought tolerance of two grape cultivars under *in vitro* conditions.

Materials and Methods

The shoots of two grapevine varieties (White Seedless, Flame Seedless) were excised from rooted cuttings of Malayer Grape Research Center, Malayer, Iran. The shoots were placed under tap water for 1 h and submerged in a 1% bleach solution containing 5.5% sodium hypochlorite for 10 min. Shoots were rinsed three times by sterile distilled water. Then, single-node explants (10-15 mm in length) were prepared and transferred to 140 mm × 75 mm glass jars containing 100 ml of the Murashige and Skoog (MS) basal medium. The medium was supplemented with 2 mg/l N6-benzyladenine (BA), 0.2 mg/l naphthalene acetic acid (NAA), 30 g/l sucrose, 7 gr agar and 200 mg/l activated charcoal. The pH of the media was adjusted to 5.7 ± 0.1 with 0.1 N HCl or NaOH before sterilization (autoclaving at 121 °C for 15 min). Cultures were maintained at 24 ± 1 °C and 16/8 h light/dark photoperiod using cool-white fluorescent lights at 57 μmol.m^{-2}.s^{-1}. The uniformly developed explants were transferred to the same medium having PEG 6000 concentrations 0, 0.5, 1, and 2%. PEG was added to the medium before the pH adjustment, while activated charcoal was added after the pH adjustment. The explants were maintained under similar conditions as described above for 30 days. At the end of the experiment, fresh weight (FW), dry weight (DW), relative water content (RWC), chlorophyll content, proline, and soluble carbohydrates were measured. The length of the main branch and the number of leaves were measured by a ruler.

To measure the RWC of the leaves, 10 leaf discs with a diameter of 7 mm were weighed (FW). Then, the discs were hydrated until saturation for 4 h at 4 °C in darkness, and their turgor weight (TW) was measured. To obtain DW, the leaf discs were dried in an oven at 70 °C for 48 h. RWC was determined by the following formula:

$$\text{RWC (\%)} = [(\text{FW} - \text{DW}) / (\text{TW} - \text{DW})] \times 100$$

Proline and soluble carbohydrate content were measured using 100 mg leaf samples based on Paquin and Lechasseur (1979). The absorbance of proline and soluble carbohydrate content was measured at 515 and 625 nm, respectively, using a spectrophotometer (Carry100, Variyan, USA). L-proline and glucose were used as the standard.

The chlorophyll content was measured in 100-mg leaf samples based on Gross (1991) as follows:

$$\text{Total chlorophyll (gr/l)} = (\text{OD}_{645} \times 0.0202) + (\text{OD}_{663} \times 0.00802)$$

$$\text{Chlorophyll a (gr/l)} = (OD_{645} \times 0.0127) + (OD_{663} \times 0.00269)$$

$$\text{Chlorophyll a (gr/l)} = (OD_{645} \times 0.0229) + (OD_{663} \times 0.00468)$$

The absorbance was measured at 645 and 663 nm using a spectrophotometer (Carry100, Variyan, USA).

The experiment was conducted as factorial [four concentrations of PEG 6000, i.e., 0, 0.5, 1, and 2% (w/v) and two grape cultivars] using a completely randomized design with four replications per treatment. Statistical data analysis was carried out using the GLM procedure of the SAS 9.1 software. Assumptions of the analysis of variance were fulfilled by the square root transformation. After carrying out the analysis of variance, treatment means were compared by Duncan's multiple range test ($p \leq 0.05$) method. Figures were drawn by Excel.

Results

Effects of PEG and cultivar on growth characters of the grapevine explants

All growth characters of the grapevine explants were significantly affected by cultivars, PEG, and their interaction, except the cultivar × PEG interaction for the number of leaves (Table 1). The Flame Seedless cultivar was significantly higher than the White Seedless in shoot length, the number of leaves per shoot, FW, and DW, on the average of the PEG levels. Also, it had a significantly higher shoot length at the control and 1% PEG conditions. At the 2% PEG, the shoot length of the explants was significantly declined in the Flame Seedless variety as compared to the control but the decline was not significant at the 1% PEG level (Table 2; Figures 1A and 1B). Although the shoot length was also declined in the White Seedless variety at 2% PEG, the change was not significant (Table 2; Figure 1C). On the average of two varieties, the lowest and highest shoot length belonged to the 2% (w/v) PEG in the media and the control (0.31 cm and 1.30, respectively). The number of leaves per shoot was also reduced at 1 and 2% PEG levels but the reduction was not significant in the Flame Seedless cultivar (Table 2). The lowest and highest number of leaves were obtained with the 2% (w/v) PEG and the control (1.18 and 3.80, respectively). At the PEG concentration of 0.5%, the FW of the explants decreased but at the PEG concentration of 2%, DW increased significantly.

Effects of PEG and cultivar on biochemical and physiological characters of the grapevine explants

There were significant differences between cultivars in terms of proline, soluble carbohydrates, and the chlorophyll b content. Also, RWC, proline, and chlorophyll a were affected significantly by PEG. The cultivar × PEG interaction was only significant for RWC and proline (Table 3).

Increasing PEG concentration to the 2% level in the media led to the significant proline accumulation in the leaves of the grape explants averaged over two cultivars. The lowest and highest proline content (178.10 and 317.28 µmol/gr leaf FW, respectively) was due to the concentration of 0.5 and 2% (w/v) PEG in the media, respectively. Among the cultivars tested, White Seedless had the highest proline content

Table 1. Analysis of variance of the effects of PEG and cultivar on growth characters of the grapevine explants

SOV	df	Mean squares			
		Length of the main branch	Number of leaves	Fresh weight	Dry weight
Cultivars	1	5.85**	7.61**	0.0052**	0.0013**
PEG	3	0.82**	3.46**	0.014**	0.0020**
Cultivars × PEG	3	1.00**	0.53^{ns}	0.052**	0.0021**
Error	55	0.16	0.60	0.00055	0.00010
CV (%)	-	42.09	69.64	32.91	29.13

^{ns,**}non-significant and significant at $p \leq 0.01$, respectively

Table 2. Means of growth characters of grapevine explants as affected by drought stress (PEG) and cultivar

Factors and treatments	Shoot length (cm)	Number of leaves per shoot	Fresh weight (gr)	Dry weight (gr)
Variety				
Flame Seedless	1.34a	2.93a	0.044a	0.019a
White Seedless	0.48b	1.61b	0.039b	0.017b
PEG level				
Control	1.30a	3.80a	0.057a	0.017b
0.5% PEG	1.15a	2.50a	0.028b	0.015b
1% PEG	0.93a	1.75b	0.039ab	0.018b
2% PEG	0.31b	1.18b	0.044ab	0.022a
Treatment				
Control (0 PEG) - Flame Seedless	1.93a	4.37a	0.067a	0.016ab
0.5% PEG - Flame Seedless	1.12bc	2.87a	0.021b	0.013b
1% PEG - Flame Seedless	1.87ab	2.37a	0.039ab	0.021ab
2% PEG - Flame Seedless	0.47cd	2.12a	0.050ab	0.028a
Control - White Seedless	0.57cd	3.14a	0.045ab	0.019ab
0.5% PEG - White Seedless	0.75c	2.12a	0.036b	0.018ab
1% PEG - White Seedless	0.43d	1.12b	0.038b	0.015b
2% PEG - White Seedless	0.18d	0.25b	0.037b	0.016ab

Means with the same letter within each category are not significantly different based on Duncan's multiple range test ($p \leq 0.05$).

(343.74 µmol/gr leaf FW). Also, the highest proline content (524.20 µmol/gr leaf FW) was obtained in the White Seedless explants at the PEG concentration of 2% (Table 4). On the other hand, according to Table 4, Flame Seedless had higher soluble carbohydrates content than the White Seedless cultivar.

Table 4 shows a significant reduction in RWC under drought stress. The lowest and highest RWC (29.00 and 57.83, respectively)

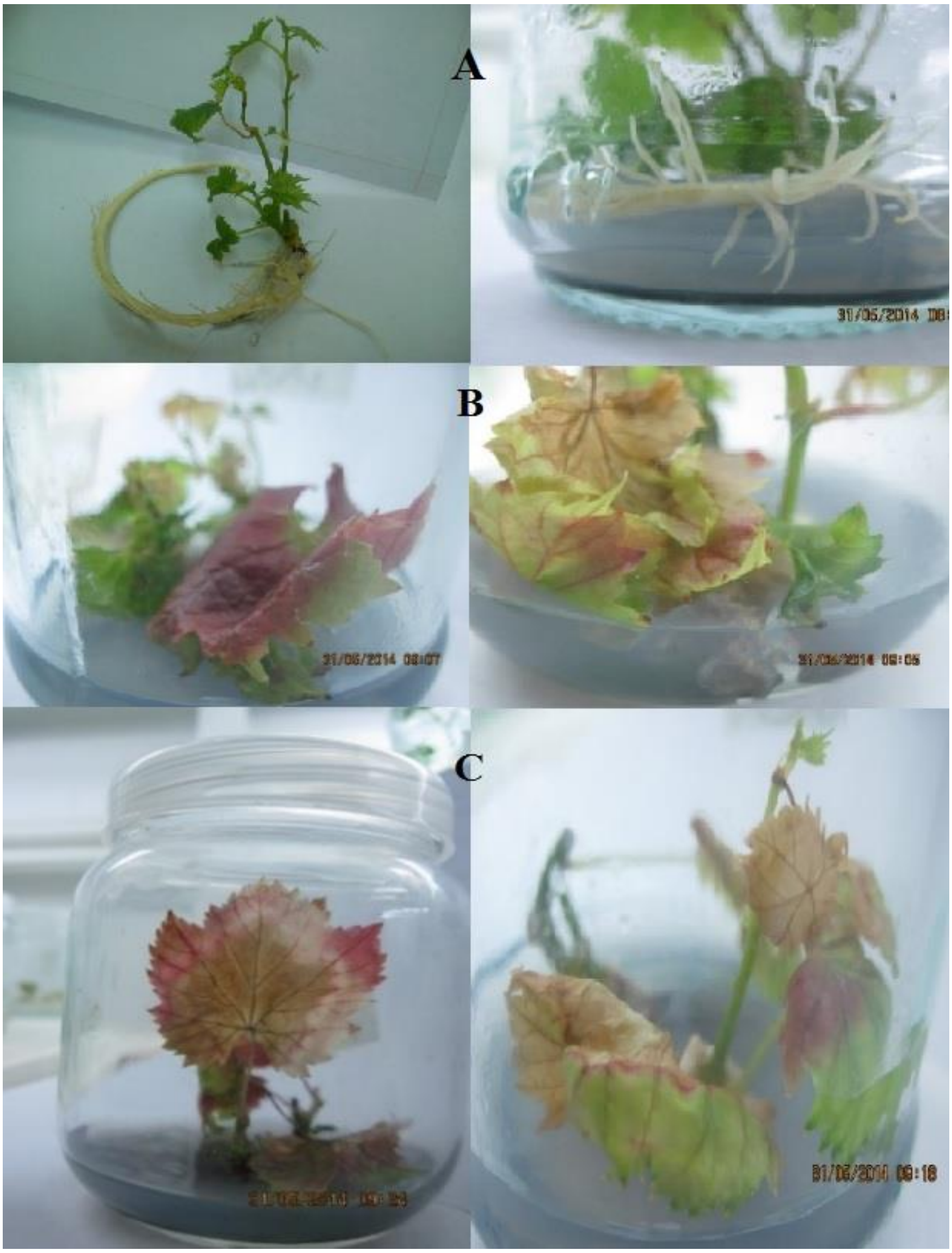

Figure 1. Explants of grapevine. A) The Flame Seedless cultivar at no PEG; B) The Flame Seedless cultivar at 2% (w/v) PEG; C) The White Seedless cultivar at 2% (w/v) PEG

was due to the concentration of 0.5% (w/v) PEG in the media and the control. No significant difference was observed between the two cultivars for RWC (Table 4). However, there was a significant interaction between cultivar × drought stress. The lowest and highest RWC (19.99 and 67.14) belonged to the Flame Seedless explants at no PEG and 0.5% PEG, respectively. In White Seedless, RWC remained stable with the increase in the stress level (Table 4).

Table 3. Analysis of variance of the effects of PEG and cultivar on biochemical and physiological characters of the grapevine explants

SOV	df	Mean squares					
		Relative water content	Proline	Soluble carbohydrates	Total chlorophyll	Chlorophyll a	Chlorophyll b
Cultivars	1	1.77^{ns}	757875**	6.20**	0.23^{ns}	0.11^{ns}	0.55**
PEG	3	22.30**	129383**	0.64^{ns}	0.26^{ns}	0.11*	0.16^{ns}
Cultivars × PEG	3	6.54*	129357**	0.11^{ns}	0.04^{ns}	0.02^{ns}	0.02^{ns}
Error	50	1.65	949	0.41	0.27	0.031	0.06
CV %	-	22.09	13.59	27.15	33.95	32.02	35.26

^{ns,*,**}non-significant and significant at $p \leq 0.05$ and $p \leq 0.01$, respectively

Table 4. Means of the biochemical and physiological characters of grapevine explants as affected by drought stress (PEG) and cultivar

Factors and treatments	Relative water content (%)	Proline (µmol/gr leaf FW)	Soluble carbohydrates	Total chlorophyll	Chlorophyll a	Chlorophyll b
			(mg/gr of fresh weight)			
Cultivar						
Flame Seedless	37.59a	139.34b	7.78a	1.09a	0.39a	0.71a
White Seedless	40.37a	343.74a	4.39b	0.80a	0.26a	0.52b
PEG						
Control	57.83a	217.41bc	7.96a	1.55a	0.54a	0.98a
0.5% PEG	29.00b	178.10c	5.82a	0.86a	0.31b	0.56b
1% PEG	38.60b	228.74b	5.85a	0.77a	0.28b	0.50b
2% PEG	31.07b	317.28a	5.43a	0.77a	0.24b	0.53b
Cultivar × PEG						
Control - Flame Seedless	67.14a	140.74d	9.21a	1.64a	0.57a	1.03a
0.5% PEG - Flame Seedless	19.99d	134.28d	6.94abc	0.85bc	0.30ab	0.54ab
1% PEG - Flame Seedless	39.00bc	146.12d	8.01ab	0.93bc	0.34ab	0.60ab
2% PEG - Flame Seedless	22.33d	136.23d	7.00abc	0.95bc	0.34ab	0.62ab
Control - White Seedless	47.19b	319.64b	5.97abcd	1.33ab	0.47a	0.85a
0.5% PEG - White Seedless	38.02bc	221.92c	4.69bcd	0.88bc	0.32ab	0.58ab
1% PEG - White Seedless	38.14bc	323.16b	3.70cd	0.64c	0.22b	0.40b
2% PEG - White Seedless	38.73bc	524.20a	3.63d	0.61c	0.15b	0.46b

Means with the same letter within each category are not significantly different based on Duncan's multiple range test ($p \leq 0.05$).

The PEG stress decreased both Chlorophyll a and Chlorophyll b contents significantly. Although the Flame Seedless cultivar had higher chlorophyll a and chlorophyll b than the White Seedless the difference between the two cultivars was not significant in terms of the Chlorophyll a content.

Effects of PEG and cultivar on callus production of the grapevine cultivars

Flame Seedless at the 1% PEG level produced the highest amount of callus (87.5%). The lowest percentage of callus (6.2%) was obtained in the White Seedless explants under no PEG treatment (Table 5). Figure 2 shows the red callus and red

Table 5. Percentage of callus production in two cultivars of grapevine at different PEG concentrations

PEG	Callus (%)	
	Flame Seedless	White Seedless
Control	18.7	6.2
0.5% PEG	12.5	12.5
1% PEG	87.5	12.5
2% PEG	43.7	25

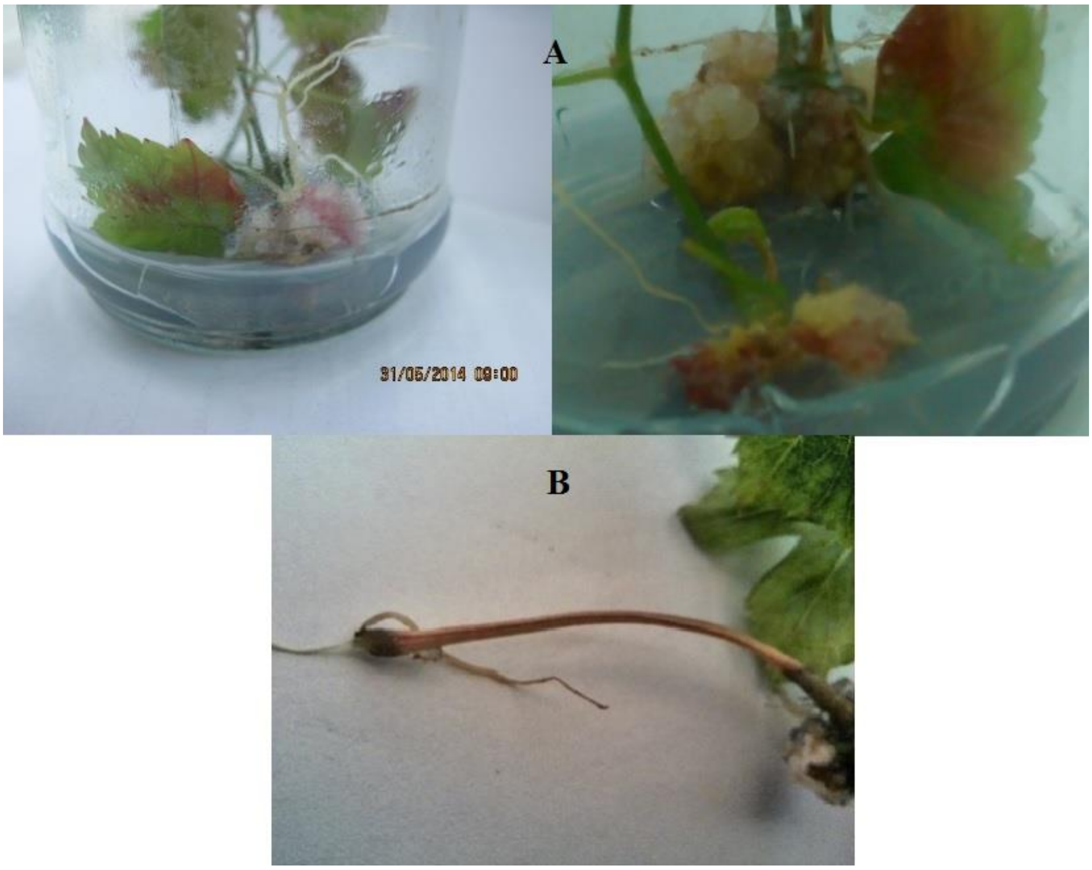

Figure 2. A) Red callus production at the 1% PEG level of the Flame Seedless explants; B) Red root production at the 1% PEG level of the Flame Seedless explants

root production of the Flame Seedless explants at the 1% PEG level of stress.

Discussion

This study investigated two grape cultivars *in vitro* at PEG-induced drought stress. Attempts have been made to select abiotic stress-tolerant plants at *in vitro* conditions in a wide range of plant species, including cereals, vegetables, fruits, and other commercially important plant species (Rai *et al.* 2011; Bigdeloo *et al.* 2018). Previous studies on *in vitro* propagation of the *Vitis* genus have shown that the rate of succession in each culture stage depends on the type of the genotype (Novak and Jůvova 1982; Bajaj 1986; Reisch 1986; Péros *et al.* 1998; Smerea *et al.* 2010;

Alizadeh *et al.* 2010; Eftekhari *et al.* 2012). In the present study, the Flame Seedless and White Seedless varieties were successfully established in the MS basal medium. However, different *in vitro* responses were observed between these two varieties. Péros *et al.* (1998) compared micropropagation responses of several grape varieties and found highly significant differences in terms of stem length and the number of roots and nodes. In several plant species, the differences regarding *in vitro* responses among genotypes have been suggested to be related to differences in endogenous hormone contents (Looney *et al.* 1988; Alvarez *et al.* 1989; Grönroos *et al.* 1989; Pourasadollahi *et al.* 2019). The same assumption may be adopted to explain the great variability between *V. vinifera* varieties (Péros *et al.* 1998).

Growth characters of the explants such as FW, shoot length, and the number of leaves per shoot were significantly reduced by increasing the PEG concentration in the media. Oukabli *et al.* (2008) also reported a limited growth under drought stress. PEG reduces water potential and simulates the drought conditions in the media without exerting any toxic effects or absorption by plants (Rumbaugh and Johnson, 1981; Kent and Lauchli, 1985; Bigdeloo *et al.,* 2018).

Some physiologists believe that RWC is a valuable index to evaluate the water content of plant tissues (e.g. Kramer 1983). Most studies have shown a reduction in RWC in response to drought stress (Augé *et al.* 2003; Liu *et al.* 2008; Sarvari *et al.* 2017). In the present study, RWC was reduced as PEG concentration increased in the media. In the White Seedless cultivar, RWC remained stable after increasing the stress level, and the reduction was only 19 to 18% compared to the control. On the other hand, the Flame Seedless cultivar had significantly higher RWC than the White Seedless at normal conditions but its RWC was sharply reduced when the PEG stress was imposed. This shows that the White Seedless cultivar managed the PEG stress better than the Flame Seedless considering RWC.

A reduction in leaf water content under drought conditions leads to the accumulation of osmolytes, such as proline, in the leaves. Proline accumulation has been frequently reported in many plants or tissues in response to other abiotic stresses. However, the precise role of proline accumulation is still unclear. It may act as an osmoregulator, an osmo-protector, or a regulator of cellular redox potential (Ozden *et al.* 2009). In the current study, the leaf proline content was variety- dependent. It was not significantly increased with an increase in the PEG concentration in the Flame Seedless cultivar but a significant increase was observed in the White Seedless explants at the 2% PEG level as compared to the control (Table 2). Other investigators also reported the drought stress-induced proline accumulation in the field and *in vitro* experiments (Sivritepe *et al.* 2008; Abhari and Gholinezhad 2019). Taylor (1996) showed that declining water content in plant tissues triggers proline accumulation. Also, Trotel *et al.* (1996) indicated the proline accumulation in tissues once they were put under salt stress conditions. Solomon *et al.* (1994) indicated that proline could protect cell membranes under stress conditions. Türkan *et al.* (2005) and Verslues *et al.* (2006) also suggested that proline might act as a reactive oxygen species scavenger and a cell membrane stabilizer, thereby protecting cells against oxidative stress and dehydration. These findings highlight the hypothesis that proline accumulation may protect cells against environmental stresses (Sivritepe *et al.* 2008).

The results revealed that PEG-induced drought stress reduced the chlorophyll a content. However, proline contents increased with an increase in PEG concentration. The lowest chlorophyll a content and highest proline content were obtained at the concentrations of 2% (w/v) PEG in the White Seedless explant (Table 2). This reduction in chlorophyll a content during stress can be attributed to increasing proline content, which is, in turn, due to a common precursor, i.e., glutamate (Le Dily *et al.* 1993).

In the present study, PEG application did not significantly change the soluble carbohydrates in the explants. However, the soluble carbohydrates of the Flame Seedless cultivar were significantly higher than the White Seedless cultivar when PEG was imposed at the 1 and 2% concentrations. Also, relative to the respective controls, the soluble carbohydrates of the Flame Seedless cultivar were 25% higher than the White Seedless at 1% PEG and 14% higher at 2% PEG. This indicates that Flame Seedless endures the PEG stress by the management of its soluble carbohydrates. Accumulation of soluble carbohydrates during drought stress conditions has been reported in grape (Patakas and Noitsakis 2001), strawberry (Zhang and Archbold 1993), and *Calendula officinalis* L. (Khalilzadeh *et al.* 2020). According to Hoekstra *et al.* (2001), the accumulation of soluble carbohydrate content is closely related to drought resistance in plants. Glucose is a precursor of anthocyanin, whose increasing concentrations can be observed during stressful situations (Simões *et al.* 2009).

Conclusions

Based on the growth characters of the explants, the Flame Seedless cultivar was more vigorous than White Seedless and better managed its shoot length, number of leaves per shoot, DW, chlorophyll a, chlorophyll b, soluble carbohydrates, and callus production of the explants at 1% PEG stress level. Although the White Seedless cultivar was not more vigorous than Flame Seedless but showed significantly higher proline content, non-significant reduction in RWC, and a slightly lower reduction in shoot length and FW of the explants at 2% PEG as compared to the control. It seems that both varieties succeeded in dealing with stress with their unique mechanisms. Osmoregulation is a type of stress avoidance mechanism. Increased proline content and non-significant reduction of RWC at 2% PEG in the White Seedless cultivar and lower non-significant reduction of soluble carbohydrates content in the Flame Seedless cultivar as defense factors of the plant tissues may have contributed to the drought stress tolerance of these grapevine cultivars.

Conflict of interest

The authors declare that they have no conflict of interest with any people or organization concerning the subject of the manuscript.

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ارزیابی واکنش دو رقم انگور (*Vitis vinifera* L.) به تنش خشکی در شرایط درون درون شیشه‌ای

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چکیده

تنش‌های غیر زنده تهدیدی بزرگ برای کشاورزی به حساب می‌آیند. بنابراین، تولید گیاهان متحمل به تنش از اهمیت زیادی در افزایش بهره‌وری برخوردار است. انگور (Vitis vinifera L.) یکی از گیاهان خوراکی مهم تحت کشت در سراسر جهان است. به منظور بررسی واکنش دو رقم انگور، بیدانه سفید و فلیم سیدلس، به تنش خشکی، آزمایشی در شرایط درون شیشه‌ای طراحی شد. عامل دوم شامل چهار غلظت ۰، ۰/۰۵ ، ۱ و ۲ درصد پلی اتیلن گلیکول ۶۰۰۰ بود. این غلظت‌ها به ترتیب معادل ۰، ۰/۰۳۵-، ۰/۰۷- و ۰/۱۴- بار پتانسیل آب بودند. ریزنمونه‌های تک گره‌ای انگور رشد یافته در محیط موراشیگ و اسکوک با تنظیم کننده‌های بنزیل آدنین (۲ میلی گرم در لیتر)، نفتالین استیک اسید (۰/۲ میلی گرم در لیتر)، ساکارز (۳۰ گرم در لیتر)، آگار (۷ گرم) و ذغال فعال (۲۰۰ میلی گرم در لیتر)، پس از استقرار کامل به محیط کشت مشابه ولی با غلظت‌های مختلف پلی اتیلن گلیکول به مدت ۳۰ روز منتقل شدند. نتایج نشان داد که رقم فلیم سیدلس از ویژگی‌های رشد بهتری نسبت به رقم بیدانه سفید در متوسط غلظت‌های PEG برخوردار بود. فلیم سیدلس همچنین تنش خشکی را از نظر طول شاخساره، تعداد برگ در شاخساره، وزن خشک ریزنمونه، کلروفیل a، کلروفیل b و کربوهیدرات‌های محلول به طور موثرتر از رقم بیدانه سفید مدیریت کرد و از درصد بالای کالوس (۸۷/۵ درصد) در غلظت ۱ درصد PEG برخوردار بود.. اگرچه رقم بیدانه سفید از نظر ویژگی‌های رشدی قوی‌تر از فلیم سیدلس نبود، ولی محتوای پرولین بالاتر معنی‌دار، کاهش غیرمعنی‌دار در محتوای آب نسبی و کاهش اندکی در طول ساقه و وزن تر ریزنمونه‌ها در غلظت ۲ درصد PEG نسبت به شاهد نشان داد. به نظر می‌رسد هر دو رقم انگور با سازوکارهای ویژه خود موفق به مقابله با تنش خشکی PEG شده‌اند.

واژه‌های کلیدی: انگور؛ پلی اتیلن گلیکول؛ تنش خشکی؛ کشت درون شیشه‌ای