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Comparative study of two pear cultivars to PEG-induced osmotic stress

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Abstract
We studied the effect of polyethylene glycol (PEG)-induced osmotic stress on pear cultivars (Harrow Sweet and Bartlett) under *in-vitro* culture conditions. Explants were cultured in QL medium containing 4% and 8% PEG_{6000} using a factorial experiment based on completely randomized design. The medium without PEG was considered as control. Drought injury index, which was calculated based on morphological disorders, increased at 8% PEG nearly to 2.00 and 1.50 units in CVs. Bartlett and Harrow Sweet, respectively. Owing to osmotic stress, the increase of malondialdehyde was accompanied with the reduction of cell membrane stability index in both cultivars. Total phenolic components and antioxidant activity in leaves increased significantly in response to application of 4% and 8% PEG. However, the severity of increase was higher in CV. Harrow Sweet. It was revealed that CV. Harrow Sweet had higher tolerance to osmotic stress than Bartlett. Moreover, the parameters related to oxidative damages and ROS scavenging capacity were more descriminant against osmotic stress under *in vitro* system.

Keywords: Drought stress; *In-vitro* culture; Proline; *Pyrus communis*; Superoxide dismutase

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Introduction

Pear (*Pyrus communis* L.) is an important fruit tree species which is often cultivated in semiarid areas where its growth and productivity are influenced by water scarcity. The responses of pear mature tree to water deficit stress have been investigated in the field condition (Lopez *et al.* 2013), but little information exists on the performances of pear explants in response to osmotic stress under *in-vitro* condition. There are close correlations between cellular level and whole plant responses to stresses, hence, researchers use *in-vitro* culture techniques to evaluate the physiological and biochemical parameters of plant growth in response to abiotic stresses. Tissue culture techniques have the advantages of saving time and space as well as controlling environmental factors and experimental treatments. The effects of *in-vitro* induced drought stress have been reported on several plants (Karimi *et al.* 2012; Rao and Jabeen 2013). Shekafandeh and Hojati (2012) evaluated *in-vitro* responses of two fig CVs. (Sabz, Siah) in MS media containing four levels of polyethylene glycol. The results showed that 'Siah' was more sensitive to drought than 'Sabz'. Kim *et al.* (2011) found differential response of pear rootstocks to drought stress under *in-vitro* condition using polyethylene glycol (PEG) treatment. Polyethylene glycol, mainly PEG_{6000}, reduces water potential via imposing high water osmotic condition in culture media without any toxic

effects or absorption by explants (Michel and Kaufmann 1973) and its growth and development (Farooq *et al.* 2009).

From physiological point of view, relative water content (RWC), membrane stability index (MSI), chlorophyll (Chl) and malondialdehyde (MDA) contents and total phenolic compounds (TPC) are useful factors generally used to study plant cell responses to osmotic stress (Farooq *et al.* 2009). A common effect of water inaccessibility to plant cells is to cause oxidative damage and generation of reactive oxygen species (ROS) which results in lipid peroxidation (Gill and Tuteja 2010), chlorophyll loss and reduction of membrane stability (Basu *et al.* 2010). To alleviate injuries from ROS, plants have developed an antioxidant defence system that includes non-enzymatic compounds like phenolic compounds, carotenoids, flavonoids and enzymatic system such as superoxide dismutase and peroxidase (Agarwal and Pandey 2004). Moreover, the accumulation of low molecular weight organic solute compounds, such as proline, is another mechanism that many plant species use to alleviate the adverse effects of abiotic stress (Mahajan and Tuteja 2005).

To the best of our knowledge, no study has investigated the physiological aspects of pear, especially a comparative study between cultivars, in response to drought or osmotic stresses under *in-vitro* culture condition. Thus, there is the need to shed more light on various physiological and biochemical mechanisms on how *in-vitro* pear explants response to osmotic stress conditions and the role they play with regards to differential display of stress tolerance between cultivars. To achieve this goal, we conducted PEG-induced osmotic stress under *in-vitro* conditions on pear explants of CVs. Harrow Sweet and Bartlett.

Materials and Methods

Plant materials and growth conditions

The *in-vitro* propagated pear plantlets of CVs. Harrow Sweet and Bartlett were provided by 'Seed and Plant Improvement Institute' (Karaj, Iran). In order to proliferate the plantlets to the number needed, well growing explants were subcultured in solid QL (Quoirin and Lepoivre 1977) medium aseptically and maintained in growth chamber at 25±1 ◦C under 16 h photoperiod. The medium consisted of the QL basal medium containing 7.4 g/L Na_2EDTA (Merck), 5.57 g/L Fe_2So_4 (Merck), 30 g/L sucrose (Merck), and 6.5 g/L agar (Sigma). The medium was supplemented with 5 mg/L BA, 1 mg/L IAA, 20 mg/L glycine, 5 mg/L nicotinic acid, 5 mg/L pyridoxal hydrochloride, 1 mg/L thiamine and 100 mg/L myo-inositol. Five well-grown plantlets, almost uniform in size and about 4 cm in length, were placed in each glass jar as an experimental unit provided with 50 ml medium containing different levels of PEG (4%, 8%). According to Michel & Kaufmann (1973) the osmotic potential of medium resulted from applied PEG percentages (4%, 8%), corresponded to -0.03 and -0.10 MPa, respectively. The medium without PEG was considered as control.

Morphological and growth attributes

Six weeks after inoculation of explants into the media, shoot length (Sh. L), relative growth rate (RGR) and fresh weight increase (FWI) were measured. The explants were scored for visible symptoms of drought injury on a scale of 1 - 4 as

follows: no injury (1), browning on shoot-tips and leaf edges (2), necrosis on the whole leaf or on part of the stem (3) and whole explants dead (4). Then, drought injury index (DII) was calculated according to the following equation (Sivritepe *et al.* 2008).

$$DII = \sum(n_i \times i) / N$$

where n_i is the number of explants receiving the mark 'i' (from 1 to 4) and N is the total number of explants in each PEG concentration.

Physiological characteristics

Chlorophyll and proline contents were determined via the methods described by Arnone (1949) and Bates *et al.* (1973), respectively. Relative water content (RWC) of leaf samples was determined according to Segura-Monroy *et al.* (2015). Electrolytic leakage (EL) was assessed according to the method described by Liu *et al.* (2004) with some modifications. Membrane stability index (MSI) was determined by recording the electrical conductivity of leaf in distilled water and calculated using the following equation (Sairam *et al.* 2002).

$$MSI = [1 - (EC_1 / EC_2)] \times 100$$

where EC_1 and EC_2 are the initial and final electrical conductivity of samples, respectively.

Thiobarbituric acid test, which determines malondialdehyde (MDA) as a product of lipid peroxidation, was used to determine cell membrane lipid peroxidation (Heath and Packer 1968). Total phenolic compounds (TPC) was evaluated by the Folin-Ciocalteau method described by Khoyerdi *et al.* (2016). The DPPH (1,1-diphen l-2-picrylhydrazyl) radical scavenging activity was evaluated according to the method detailed by Sharififar *et al.* (2009). The activities of superoxide dismutase (SOD) and peroxidase (POD) enzymes were measured as described by Li *et al.* (2011).

Statistical analysis

The experiment was carried out as factorial based on a completely randomized design. Analysis of variance was performed using SAS program. Significant differences between means were determined by Duncan's multiple range test.

Results

The 4% PEG and resulted osmotic stress caused physiological disorders, necrosis and abscission of leaves in both pear cultivars. At the 8% of PEG these symptoms were accompanied with considerable dieback of shoot especially in CV. Bartlett (Figure 1). DII, which was calculated based on morphological disorders, showed a difference between two cultivars. This index increased at 8% PEG nearly to 2.00 and 1.50 units in CVs. Bartlett and Harrow Sweet, respectively (Figure 2A). Application of PEG resulted in a significant decrease in shoot length and the lowest shoot length (3.26 cm) was observed in CV. Bartlett at 8% PEG (Figure 2B). FWI was negatively influenced by osmotic stress in both cultivars. However, the reduction of FWI at 8% PEG in CV. Bartlett was approximately two times more than CV. Harrow Sweet in comparison to the control medium (Figure 2C). The growth of pear explants, indicated as RGR, decreased by increasing of PEG in the culture medium. This

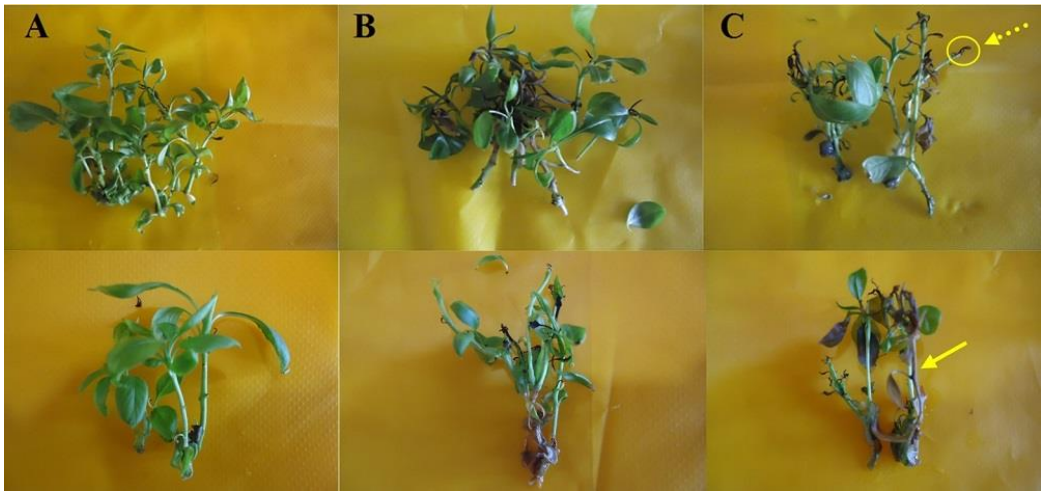

Figure 1. Effect of PEG_{6000}-induced osmotic stress on the growth of pear explants of CV. Harrow Sweet (the top row) and CV. Bartlett (the bottom row) in QL medium. Explants were grown for six weeks at the control medium (A), medium containing 4% (B) and 8% (C) PEG_{6000}. Dashed and solid arrows show necrosis of shoots tip in 'Harrow Sweet' and necrosis of whole shoot in 'Bartlett', respectively.

reduction was highest at 8% PEG. That was almost 40% and 71.24% in CVs. Harrow Sweet and Bartlett, respectively compared to the control medium (Figure 2D).

The results indicated significant effects of osmotic stress treatments on leaf Chl. and proline content of pear explants (Table 1). Increasing PEG led to significant accumulation of proline in the leaves of both cultivars, and the interaction of PEG level by cultivar was not significant. The Chl. content in the leaf tissue was significantly decreased due to increasing PEG percentage. To understand how water status of both cultivars was affected by osmotic stress, we monitored RWC in leaf tissues. The results showed the significant effect of PEG-induced osmotic stress on the leaf water loss. In the culture medium containing 4% and 8% PEG, RWC in the leaves of 'Harrow Sweet' decreased 16.29% and 28%, respectively compared to the control. Similarly, this reduction in the leaves of CV. Bartlett was 11.28% and 24.5%, respectively as compared to the control (Table 1). Application of PEG significantly increased EL (%) of pear explants as compared to the control medium. However, this changes in the 'Bartlett' was more pronounced than CV. Harrow Sweet (Figure 3A). In the control medium, MSI did not vary between the two cultivars. However, application of 8% PEG caused the reduction of this index at the rate of 39% and 65% in CVs. Harrow Sweet and Bartlett, respectively (Figure 3B). Lipid peroxidation level in the leaves of the two pear cultivars, measured as the MDA content, is given in Figure 3C. In each pear cultivar, MDA was significantly changed with PEG treatment. Exponential increase of MDA level was observed in the Bartlett explants in response to the application of PEG. However, MDA was less accumulated in the leaves of CV. Harrow Sweet. The results of TPC and antioxidant activity, based on DPPH, are shown in Figure 4. TPC increased significantly in both cultivars after application of

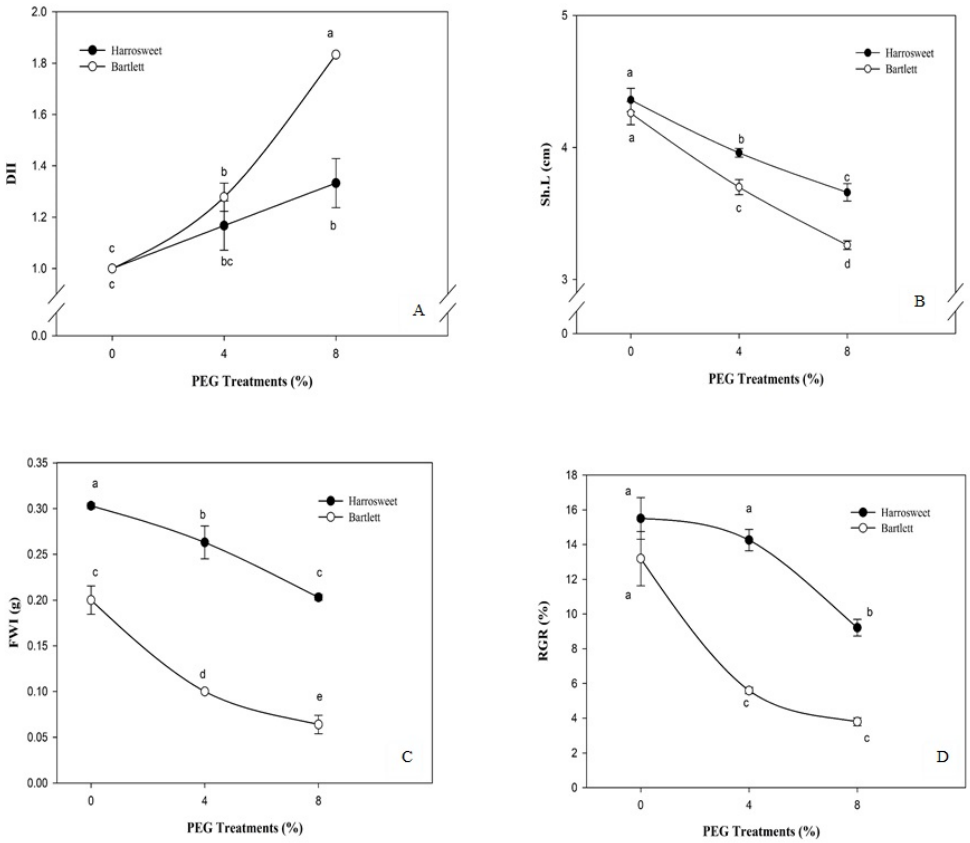

Figure 2. Influence of PEG_{6000}-induced osmotic stress on (A) drought injury index (DII), (B) fresh weight increase (FWI), (C) relative growth rate (RGR) and (D) shoot length (Sh. L) of two pear cultivar explants (CVs. Harrow Sweet and Bartlett). Bars indicate standard errors. Different letters show significant difference at $p \leq 0.05$.

PEG and reached to the highest amount in the CV. Harrow Sweet at 8% PEG (Figure 4A). From 4% to 8% PEG, DPPH showed no significant change in 'Bartlett'. However, this index increased continuously ($p \leq 0.05$) by imposing osmotic stress into the culture medium in 'Harrow Sweet' even up to 8% PEG (Figure 4B).

Activity of enzymes involved in scavenging ROS changed significantly by various levels of PEG. POD activity increased gradually by application of PEG into culture medium in both cultivars and showed 2.70 folds higher activity at 8% PEG in comparison with the control medium (Figure 4C). Also, SOD activity in the leaf tissues increased in response to PEG up to maximum amount at 4% PEG with no more increase at 8%. These increases were 1.20 and 1.80 fold higher in CVs. Bartlett and Harrow Sweet, respectively as compared to the control medium (Figure 4D).

Discussion

Application of PEG induced physiological and morphological disorders in pear explants. The severity of symptoms, chlorosis, necrosis, DII and abscission of leaves became more visible by increasing the PEG to 8%. In accordance with these results, *in-vitro* application of PEG has shown leaf necrosis in cherry explants (Sivritepe *et al.* 2008). The degradation of Chl. pigments, as was revealed in the current study, mainly

Table 1. Comparison of PEG6000-induced osmotic stress effects on different physiological traits of two pear cultivars explants.

Cultivar	PEG (%)	Chlorophyll (mg g^{-1} FW)	Proline (µmol g^{-1} FW)	Relative water content (%)
Harrow Sweet	0	0.36± 0.014a	17.35± 0.51d	92.03± 0.65a
Harrow Sweet	4	0.27± 0.003b	25.10± 0.57c	77.02±0.55c
Harrow Sweet	8	0.24± 0.004bc	30.70± 0.44a	66±0.6e
Bartlett	0	0.34± 0.015a	16.30± 0.045d	81.24±0.71 b
Bartlett	4	0.26± 0.006b	24.05± 0.51c	72.08±0.55d
Bartlett	8	0.21± 0.016c	27.75± 0.75b	61.33±0.6f

Notes: Values are means ± SE for three replications. Means with the same letter are not significantly different based on Duncan's Multiple Range Test ($p \leq 0.05$).

contributed to chlorosis and eventually necrosis of leaves under stressful osmotic condition. Similar results were reported in apple explants in the culture medium (Molassiotis *et al.* 2006). At 8% PEG, we observed whole necrosis of shoots in 'Bartlett', however, 'Harrow Sweet' showed only necrosis at shoot tips. Taken all these appearance symptoms together, one could conclude higher tolerance of 'Harrow Sweet' to osmotic stress under *in-vitro* condition.

Addition of PEG to the QL medium decreased water potential in the culture medium which decreased RGR and shoot fresh weight in the pear explants. Foliage expansion and cell division are among the most sensitive stages that are affected by water inaccessibility (Alfredo *et al.* 2004). There are ample reports dealing with the adverse effects of *in-vivo* water deficit on shoot fresh and dry weight and RGR of pear tree (Lopez *et al.* 2013) and *in-vitro* PEG-induced drought stress in cherry rootstock explants (Sivritepe *et al.* 2008).

In both pear cultivars RWC decreased by increasing PEG as result of water inaccessibility to explants. CV. Harrow Sweet had higher RWC at beginning of the experiment probably due to its genetic characteristic. This may explain why the reduction of RWC, especially from 0% to 4% PEG, in this cultivar was higher (16.29%) than 'Bartlett' (11.28%). A decrease in RWC resulted from the application of PEG under *in-vitro* culture has been noted in common fig explants (Karimi *et al.* 2012) as well as in response to water stress in pear under in-situ condition (Sharma and Sharma 2008).

Proline content of pear explants increased gradually in response to the elevation of osmotic stress. Accumulation of proline in both cultivars followed the same trend. Proline is considered to act as an osmolyte, a ROS scavenger and a molecular chaperone stabilizing the structure of proteins, thereby protecting cells from damage caused by stress (Krasensky and Jonak 2012).

Owing to PEG-induced osmotic stress damage on cell membrane, the increase in MDA was accompanied with significant increase of EL and consequently the reduction of MSI in both cultivars. EL is inversely related to cell membrane integrity and the ability to avoid or repair membrane damage has generally been correlated with abiotic stress tolerance (Tiwari *et al.* 2016). It seems that 'Harrow Sweet' maintains more cell

membrane integrity under stress condition than 'Bartlett'. This is evidenced by the lower MDA content of 'Harrow Sweet' as compared to 'Bartlett'. We noticed a coordinated change of MDA, EL and MSI in response to application of PEG on the culture medium. Bajji *et al.* (2000) also reported a close correlation among different physiological parameters such as RWC, H_2O_2 and MDA of wheat callus culture in response to PEG-induced water deficit stress.

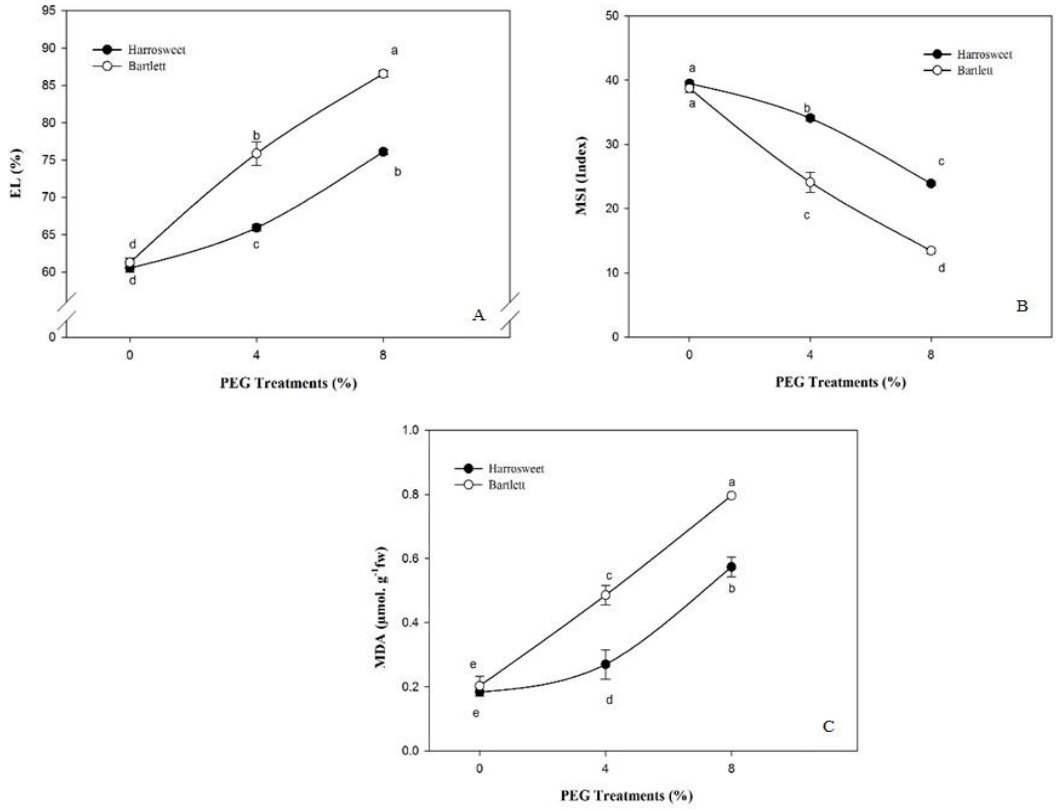

Figure 3. Influence of PEG6000-induced osmotic stress on (A) electrolyte leakage (EL), (B) membrane stability index (MSI) and (C) malondialdehyde (MDA) content of pear explants (CVs. Harrow Sweet and Bartlett). Bars indicate standard errors. Different letters show significant difference at $p \leq 0.05$.

Accumulation of TPC and increasing of antioxidant activity would be necessary to protect lipid membrane from oxidative stress in plants subjected to abiotic stress (Zhu *et al.* 2009). Increasing of enzymatic and non-enzymatic antioxidant defence capacity is quite crucial to maintain the components in their functional conformation. In our study TPC and DPPH parameters as well as antioxidant enzymes (POD and SOD) activities in leaves of pear explants raised significantly in response to application of 4% and 8% PEG. These parameters display the same trend of changes in both cultivars. However, the severity of these changes was higher in 'Harrow Sweet'. In confidence with these results, a number of studies have shown the accumulation

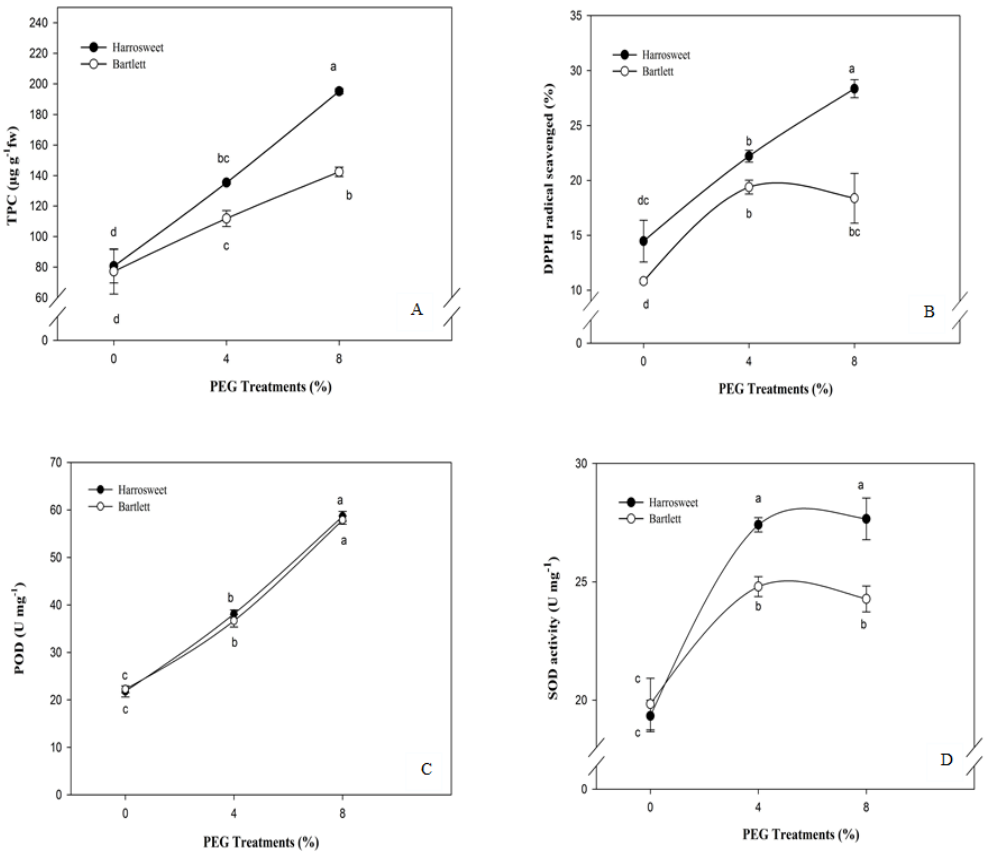

Figure 4. Influence of PEG6000-induced osmotic stress on total phenolic compounds (A), antioxidant activity (B) and superoxide dismutase activity (C) and peroxidase activity (D) of pear explants (CVs. Harrow Sweet & Bartlett). Bars indicate standard error. Different letters are significantly different ($P < 0.05$).

of antioxidant enzymes such as SOD and POD in plants' tissues stressed by salinity (Keutgen and Pawelzik 2008) and osmotic stress (Sivritepe *et al.* 2008). The difference in action capacity of SOD, but not POD, between studied pear cultivars was revealed in the current study. One possible explanation to this is that the SOD enzyme is the first line of defence against ROS and catalyzes the conversion of the superoxide anion to H_2O_2 (Gill and Tuteja 2010). In previous studies, under in-situ condition, it has been confirmed that drought-tolerant olive (Petridis *et al.* 2012) and pistachio (Khoyerdi *et al.* 2016) genotypes showed higher antioxidant defence capacity in comparison to sensitive genotypes.

Conclusion

This research revealed that the two pear cultivars showed different tolerance to stress, but the trend of changes in majority of measured characters was similar. Generally, CV. Harrow Sweet showed higher tolerance to stress than 'Bartlett'. From biochemical point of view, the attributes related to oxidative damages and ROS scavenging capacity, especially SOD activity, were more discriminant against osmotic stress in the pear explants under *in-vitro* culture system.

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مقایسه پاسخ ریزنمونه‌های دو رقم گلابی به تنش اسمزی ناشی از پلی‌اتیلن‌گلیکول

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چکیده

در این تحقیق تاثیر تنش اسمزی ناشی از کاربرد پلی‌اتیلن‌گلیکول (PEG) روی ریزنمونه‌های گلابی ارقام هروسوئیت و بارتلت در شرایط کشت درون‌شیشه‌ای مورد مطالعه قرار گرفت. ریزنمونه‌ها در محیط کشت QL حاوی ۴ و ۸ درصد PEG_{6000} در قالب آزمایش فاکتوریل بر پایه طرح کاملا تصادفی کشت شدند. محیط کشت فاقد پلی‌اتیلن‌گلیکول به عنوان شاهد در نظر گرفته شد. شاخص آسیب خشکی، که بر اساس آسیب‌های فیزیولوژیکی ظاهری تعیین شد، در تیمار ۸ درصد PEG حدود ۲ و ۱/۵ واحد به‌ترتیب در ارقام بارتلت و هروسوئیت افزایش نشان داد. تنش اسمزی سبب کاهش پایداری غشاء سلولی و متعاقباً افزایش ترکیب مالون‌د-آلدهید بافت برگ در هر دو رقم شد. مقدار فنول کل و ظرفیت آنتی‌اکسیدانی بافت برگ ریزنمونه‌ها به طور معنی‌داری در مواجهه با کاربرد ۴ و ۸ درصد PEG افزایش یافت. لیکن شدت این افزایش در رقم هروسوئیت بیشتر بود. بر اساس نتایج این آزمایش، ریزنمونه‌های گلابی رقم هروسوئیت تحمل بیشتری به تنش اسمزی نشان دادند. علاوه بر این، متغیرهای مرتبط با آسیب‌های اکسیداتیو و ظرفیت جاروبی رادیکال‌های آزاد اکسیژنی برای مطالعات مقایسه‌ای تحمل به تنش اسمزی در شرایط کشت درون‌شیشه‌ای کارآمدی بیشتری نشان دادند.

واژه‌های کلیدی: پرولین؛ تنش خشکی؛ سوپراکسید دیسموتاز؛ کشت درون شیشه‌ای؛ *Pyrus communis*