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Biochemical and Physiological Responses of Alfalfa (*Medicago sativa* L.) Cultivars to Osmotic Stress

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Abstract
In order to investigate the effects of water stress on total phenolics content, antioxidant power, β-glucosidase activity and stomatal properties of alfalfa, a factorial experiment based on randomized complete block design was carried out in 1-Lit pots containing half strength Hoagland culture medium using two cultivars of alfalfa at four osmotic pressures including 0 (control), -0.5, -1.0 and -1.5 MPa. Polyphenol oxidase activity and total phenolics were increased in both cultivars in response to osmotic stress. Although the increases in total phenolics were higher in Yazdi cultivar than Gharayonjeh but the polyphenol oxidase activity had an inverse trend, thus resulting in higher levels of phenolic compounds in Yazdi cultivar than Gharayonjeh. The β-glucosidase activity as a marker of ABA level in plant cells increased in both cultivars. Furthermore, stomatal conductance and transpiration rates decreased in response to drought stress. This means that both cultivars closed their stomata under osmotic pressure in order to reduce the transpiration, however, Yazdi cultivar was more efficient in this respect. According to our results, it can be concluded that Yazdi can be considered as a more tolerant cultivar than Gharayonjeh because of its ability to increase free ABA levels in leaves, reduce transpiration rate and accumulate antioxidant compounds.

Keywords: Alfalfa; Beta-glucosidase; Osmotic stress; Phenolic compounds

Introduction

Drought stress is one of the most common adverse environmental conditions which can reduce crop productivity seriously. The percentage of drought affected land areas has nearly doubled from 1970s to the early 2000 in the world (Isendahl and Schmidt 2006). It is estimated that the percentage of droughty terrestrial areas will redouble by the end of 21st century. Drought as a world spread problem seriously influence grain production and quality. The global climate changes with increasing population make above situation more serious (Hongbo *et al.* 2005). It has been reported that drought induced damages to the photosynthetic apparatus can be additionally enhanced by irradiation. Soil water deficit induces the leaf dehydration and leads to intensify the production of reactive oxygen species (ROS), which has direct and indirect effects on the synthesis of antioxidants and on secondary metabolism (Souza *et al.* 2004).

Plants adapt to drought by down-regulation of several physiological and biochemical processes, thereby maintaining growth even under severe water stress (Izanloo *et al.* 2008). To withstand against the stress, plants have developed a number of morphological, physiological, and biochemical responses characterized by the reduce in the water potential of plant tissues which leads to a variety of

modifications in different plant processes including accumulation of abscisic acid and osmoprotectant solutes, stomatal closure, growth inhibition, reduced transpiration and formation of radical scavenging compounds (Shao *et al.* 2009).

The great attention has attracted to the ecological roles of phenolic compounds in protecting plants from unfavorable internal or external environment (Cheng and Li 2006). Synthesis of polyphenol compounds is induced in response to biotic and abiotic stimuli such as UV-B radiation, drought, chilling, ozone, heavy metals, attack by pathogens, wounding or nutrient deficiency. Polyphenols as antioxidant compounds protect the plant against oxidative stress (Grace 2005). Phenolic compounds, including simple phenols (phenolic acids and derivatives) and polyphenols (flavonoids and polymeric compounds) play an important role in the detoxification of free radicals (Ksouri *et al.* 2007). Thus, increased synthesis of phenolic compounds is a common response to water deficit (Parida *et al.* 2004).

Abscisic acid (ABA) is a phytohormone critical for plant growth, development and adaptation to various stress conditions. In order to respond to changing physiological and environmental conditions, plants have to regulate ABA levels (Lee *et al.* 2006). In particular, cellular ABA levels increase significantly during seed maturation and under abiotic stress. The amounts of cellular ABA in a leaf cell may be increased by two different ways, the first and main way is de novo synthesis in the leaf cell and supply from roots (Nambara and Marion-Poll 2005). The second way is ABA-GE hydrolysis which may contribute to ABA homeostasis in plant cells (Chiwocha *et al.* 2005).

Selection of drought tolerant varieties would be the most economical approach to improve productivity and to reduce agricultural use of fresh water resource (Gao *et al.* 2008). This investigation was carried out to find a) water stress effects on total phenolics, polyphenol oxidase (PPO) activity, antioxidant power and β-glucosidase activity as an indicator of ABA in two alfalfa cultivars and b) to find how sensitive and tolerant cultivars respond to stress condition.

Material and Methods

Plant material

The experiment was carried out using two cultivars of alfalfa. The two cultivars, Yazdi and Gharayonje, were distinguished based on the known standards as drought resistant and sensitive cultivars, respectively (Hosseini-Boldaji *et al.* 2012). Seeds were surface sterilized with 5% sodium hypochlorite solution for 5 min and washed with sterile water three times, then were germinated under greenhouse conditions at 24±4 °C, relative humidity of 70±20% and dark condition for 5 days in a growth chamber (Garouk, model GC.400, Iran). The seedlings were transplanted to 1-L pots containing half strength Hoagland solution and were grown under controlled condition at 25±5 °C, illumination density of 400 µmol $m^{-2}s^{-1}$ with 16/8 day/night photoperiod and aerated in necessary rate for 15 days. Afterward solutions with osmotic pressures of -0.5, -1.0 and -1.5 MPa were prepared using polyethylene glycol 6000, based on the equation provided by Michel and Kaufmann (1973). Water

potential was daily adjusted by adding appropriate volume of Hoagland half-strength solution to each container (final volume 1 L). Plants were harvested 30 days after transplanting and the samples were used for later analysis.

Assay of total phenols

The concentration of total phenols was determined with Folin-Ciocalteu reagent following the colorimetric method adapted by Singh *et al.* (2002). Measurements were carried out in triplicate and calculations were based on a calibration curve obtained with gallic acid. The levels of total phenols were expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE g^{-1} DW).

DPPH· radical scavenging activity

The diphenylpicrylhydrazyl radical (DPPH·) scavenging activity was estimated according to Hanato *et al.* (1988). The dried plant extract was diluted in the pure methanol at different concentrations ranging from 1 to 250 $\mu g.ml^{-1}$, and then 2 ml was added to 0.5 ml of a 0.2 mM DPPH· methanolic solution. The mixture was shaken vigorously and left standing at room temperature for 30 min in the dark and then the absorbance was measured at 517 nm. For each dilution of the extract, the DPPH· scavenging activity was calculated as $100^* (A_0-A_1)/A_0$, where A_0 is the absorbance of the control at 30 min and A_1 is the absorbance of the sample at 30 min. The antiradical activity was finally expressed as IC_{50} ($\mu g.ml^{-1}$), the extract concentration required to cause a 50% inhibition. A lower IC_{50} value corresponds to a higher antioxidant activity of the plant extract. All samples were analyzed in three replications.

Polyphenol oxidase activity (EC 1.10.3.1)

The assay of PPO was carried out by the method of Kumar and Khan (1982) with slight modification. Assay mixture for PPO contained 2 ml of 0.1 M phosphate buffer (pH 6.0), 1 ml of 0.1 M catechol and 0.5 ml of enzyme extract. This was incubated for 5 min at 25 ^{O}C, after which the reaction was stopped by adding 1ml of 2.5 N H_2SO_4. The absorbance of the purpurogallin formed was read at 495 nm. To the blank 2.5 N H_2SO_4 was added of the zero time of the same assay mixture. PPO activity was expressed as Ug^{-1} DW (U= change in 0.1 absorbance per min).

Extraction and assay of ABA-β-D-glucosidase

For enzyme extraction, one gram of leaves was frozen into liquid nitrogen, powdered in a cold mortar and pestle and homogenized with 1 ml ice-cold solution containing 2 mM EDTA, 1 mM magnesium acetate, 2 mM DTT and 5% (w/v) insoluble PVP at 4 °C. The homogenation was centrifuged at 15,000 g for 30 min and the supernatant was used for enzyme assay immediately. The extracellular β-glucosidase activity was measured by a modified procedure based on Garcia *et al.* (1993) method. The reaction of mixture containing 100 µl of citrate buffer (250 mM, pH 6.5), 40 µl of p-nitrophenyl-β-D-glucopyranoside (pNPG; 25mM) and 10 µL enzyme was extracted. After incubation at 37 °C for 30 min, the reaction was stopped by adding 850 µL of NaOH 0.25 M. The activity of β-glucosidase was estimated spectrophotometrically

by reading the absorbance of the liberated p-nitrophenol at 405 nm. One unit (IU) of β-glucosidase activity was defined in one minute as the amount of enzyme required for releasing 1 mM p-nitrophenol under the assay conditions.

Physiological traits

The stomatal conductance and transpiration rates were measured for leaves using a portable IRGA (infra red gas analyzer, Li 6200, Licor). The measurements were taken around 12:00 AM using three leaves for each treatment and expressed as $mmol.m^{-2}.s^{-1}$.

Statistical analysis

The experiment was conducted as factorial based on randomized complete block design using two cultivars of alfalfa at four different osmotic pressures. The data reported in this study were the mean±SE using three replicates. Statistical analysis was performed by two-way analyses of variance (ANOVA) and Duncan's multiple range test ($p\leq 0.05$). Analyses were carried out by statistical analysis system software (version 9.1, SAS Institute) and graphs drew by Excel 2003.

Results and Discussion

Total phenolics content

All osmotic stress treatments caused significant increases in phenolic compounds in Yazdi cultivar as compared with the control, but those of Gharayonje were not affected significantly ($p\leq 0.05$) as shown in Figure 1. Our results coincided with Hichem *et al.* (2009) observation who reported that an osmotic stress tolerant maize cultivar showed a higher accumulation of polyphenols. Biotic and abiotic stresses increase production of free radicals and other oxidative species in plants, which respond by increasing their capacity to scavenge reactive oxygen species (ROS). Phenolics are very significant in this field. It is also proposed that under stress conditions, phenylpropanoid biosynthesis may represent an alternative pathway for photochemical energy dissipation, which has the added benefit of enhancing the antioxidant capacity of the cell (Grace and Logan 2000). Although the mechanism of phenolic compounds stimulation by osmotic stress was not investigated in the current research, previous studies have shown that most phenolic compounds are generated by the phenylpropanoid pathway, which is stimulated by biotic and abiotic stress (Kim *et al.* 2006). This observation also can be related to the fact that phenolic compounds has strong hydrophilicity with phenolic hydroxyl and other hydrophilic groups in molecules in spite of hydrophobic parts such as aromatic rings which means that phenolic compounds could hold internal water of plants and reduce water loss in the drought environment. Thus, leaves with higher phenolic compounds would lessen transpiration (Song *et al.* 2000). Our results indicated that the more tolerant cultivar (Yazdi) increased phenolics content under water stress condition and had better capacity to confront drought stress. Similar to our findings, the enhancement in phenolic contents under water stress was reported by Oh *et al.* (2010) in lettuce and Tattini *et al.* (2004) in *Ligustrum vulgare*.

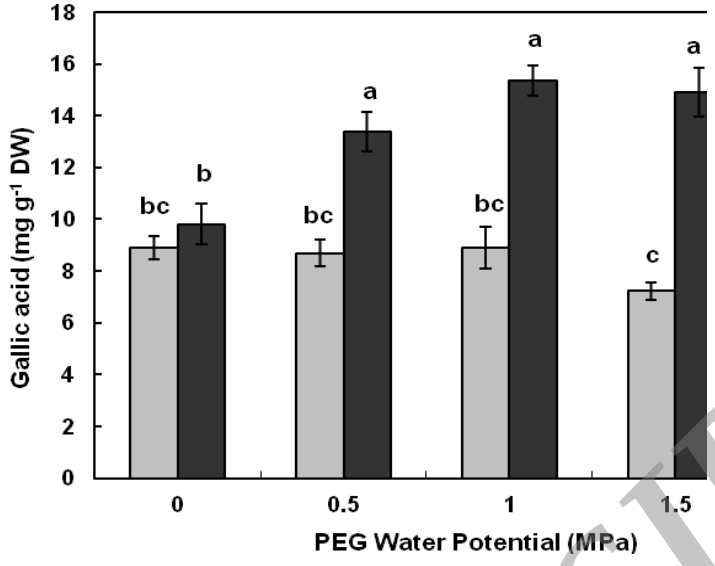

Figure 1. Changes in total phenolics of Gharayonje (gray bars) and Yazdi (black bars) cultivars incubated on PEG 6000 solutions of 0, -0.5, -1.0 and -1.5 MPa water potential. Values are expressed as means of three independent replications ± standard error. Treatments with the same lower-case letters were not significantly different based on the mean comparison by Duncan's multiple range test at p≤ 0.05.

DPPH radical scavenging activity

The free radical scavenging activity, was used to evaluate antioxidant power of the leaf methanolic extraction under optimal osmotic pressure and water deficit conditions. Antiradical properties of two cultivars are shown in Figure 2. The half inhibition concentration (IC_{50}) was decreased in Yazdi cultivar under all drought treatments in comparison with the control. But, in Gharayonjeh cultivar, it was increased under the highest osmotic pressure only and no differences were observed between other levels and the control. The principle of the antioxidant activity is the availability of electrons to neutralize free radicals such as DPPH. Substances to perform this reaction can be considered as antioxidants and therefore radical scavengers (Brand Williams *et al.* 1995). The DPPH radical scavenging activity correlated with the total phenolic content, since the r-squared values were 0.83 and 0.92 for Yazdi and Gharayonjeh, respectively (Figure 3). The phenolic compounds can limit the chlorophyll excitation during conditions unfavorable for the photosynthetic apparatus (Burchard *et al.* 2000). Moreover, phenolics have the ability to scavenge free radicals including the reactive oxygen species (Blokhina *et al.* 2002). The antioxidant activity of phenolic compounds is owed primarily to their redox properties, which enable them to act as reducing agents and hydrogen donors (Hichem *et al.* 2009). Our results indicated that phenolic compounds played an antiradical role in alfalfa under drought stress. Similar to our study other investigators have noted that cultivars with higher amounts of phenolic compounds has a better scavenging activities (Meot-Duros and Magne 2009; Gursoy *et al.* 2009).

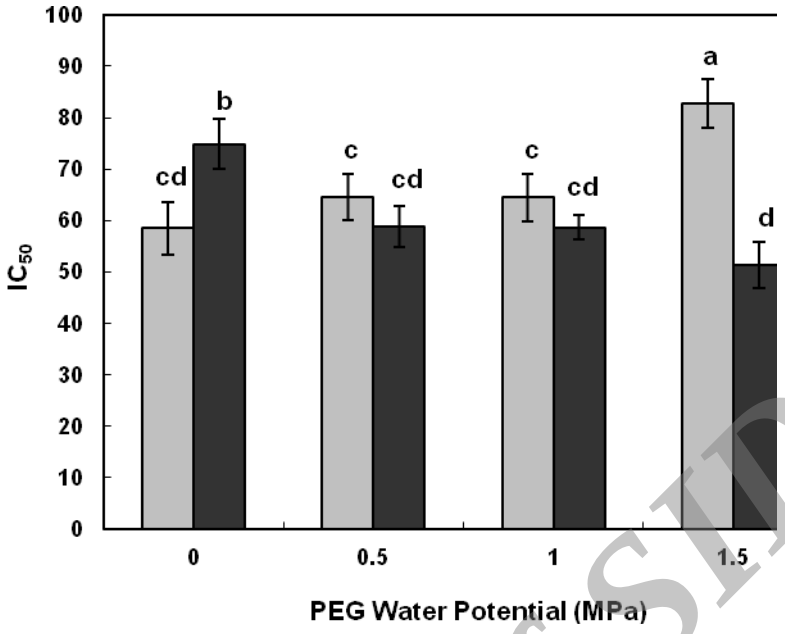

Figure 2. DPPH free radical scavenging ability of Gharayonje (gray bars) and Yazdi (black bars) cultivars incubated on PEG 6000 solutions of 0, -0.5, -1.0 and -1.5 MPa water potential. Values are expressed as means of three independent replications ± standard error. Treatments with the same lower-case letters were not significantly different based on the mean comparison by Duncan's multiple range test at p≤ 0.05.

Polyphenol oxidase activity (EC 1.10.3.1)

The results of polyphenol oxidase (PPO) activity in alfalfa cultivars under different levels of osmotic stress are shown in Figure 4. The enzyme activities were increased differently in alfalfa cultivars under osmotic stress (p≤ 0.05); the enzyme activity was higher in Gharayonjeh cultivar than Yazdi. The metabolism of phenolic compounds includes the action of oxidative enzymes such as PPO and peroxidases. Some studies have reported that the activity of these enzymes increases in response to different types of stress (Jouili and Ferjani 2003). The PPO of plants, which catalyses the oxygen dependent oxidation of phenols to quinones, has been proposed as a defense mechanism. But, in this way it has been suggested that phenol oxidation involves ROS production and could increase oxidative stress under stress conditions (Mahanil *et al.* 2008). Therefore, it could act to promote peroxidase activity, because of the generation of hydrogen peroxide during the oxidation of phenolic compounds. So, investigators believed that PPO activity can mitigate oxidative damages of stresses together with other antioxidant enzymes such as APX, POD and CAT. Our coworkers' studies on peroxidases activities in alfalfa under water stress confirmed the above suggestion. They observed that APX and POD increased significantly under water stress, although these enzymes activities were higher in Yazdi in comparison with Gharayonjeh (Hosseini Boldaji *et al.* 2012). According to the present study, the PPO activities in alfalfa cultivars were ultimate to phenolic oxidation which can raised free radical production and reduced antioxidant properties of leaf extract, so more tolerant cultivar, showed lower PPO activity under water

stress. Our results were coincided with Thipyapong *et al.* (2004) and Sunchez-Rodriguez *et al.* (2011) findings, who proposed that the higher activity of PPO in tomato, could improve resistance against water stress.

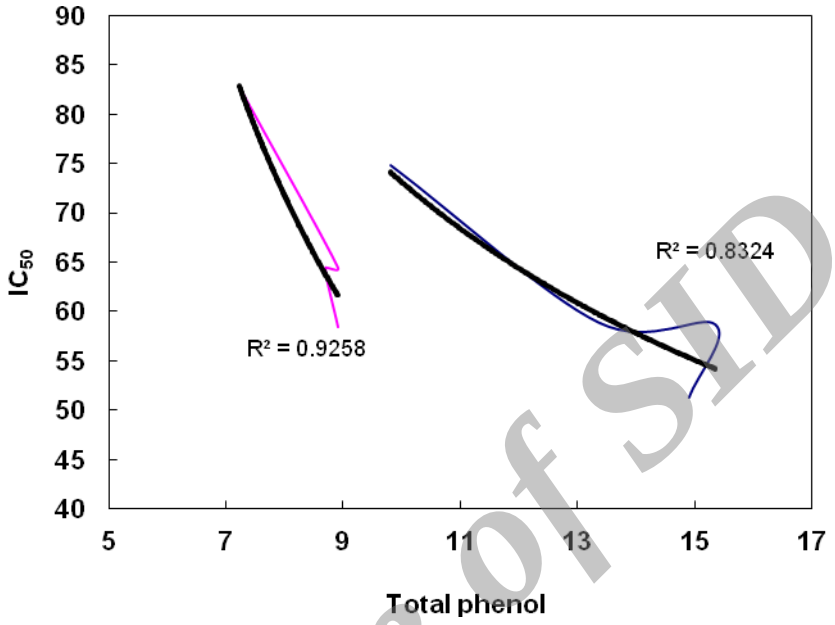

Figure 3. Relationship of total phenolics with free radical scavenging ability of Gharayonje (pink curve) and Yazdi (dark blue curve) cultivars incubated on PEG 6000 solutions of 0, -0.5, -1.0 and -1.5 MPa water potential.

β-glucosidase activity

The activity of β-glucosidase in alfalfa cultivars under water stress condition were shown in Figure 5. Results showed that the enzyme activity increased in both cultivars under water stress as compared with the control ($p \leq 0.05$). Although a significant difference in the enzyme activities of two cultivars was observed at the osmotic pressure of -1.5 MPa, at other levels of water stress, enzyme activity did not differ significantly between the cultivars ($p \leq 0.05$). The cellular ABA content is regulated via two pathways, hydroxylation and conjugation (Kushiro *et al.* 2004). ABA glucose ester (ABA-GE) is the predominant form of conjugates (Cutler and Krochko 1999). This form may contribute to ABA homeostasis in plant cells. The β-glucosidase generates ABA from ABA-GE. This pathway is ideal for rapid increase of the ABA content necessary for plants to meet physiological needs under stress condition (Lee *et al.* 2006). The results of current study represented that more tolerant cultivar (Yazdi) showed higher glucosidase activity under crucial conditions than Gharayonjeh in order to increase ABA levels and its induced stomata closure to prevent waterless through osmotic stress period.

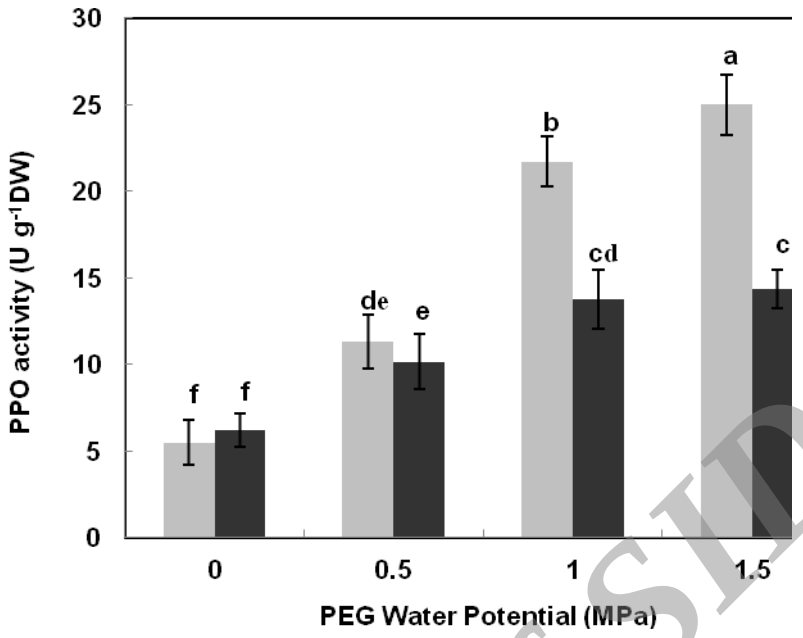

Figure 4. Polyphenol oxidase activity of Gharayonje (gray bars) and Yazdi (black bars) cultivars incubated on PEG 6000 solutions of 0, -0.5, -1.0 and -1.5 MPa water potential. Values are expressed as means of three independent replications ± standard error. Treatments with the same lower-case letters were not significantly different based on the mean comparison by Duncan's multiple range test at p≤ 0.05.

Physiological traits

Stomatal properties under drought stress were shown in Figure 6. Stomatal conductance of both cultivars reduced significantly under water stress conditions (p≤ 0.05). The reduction rate of stomatal conductance in Gharayonjeh occurred moderately whereas that of Yazdi happened severely (Figure 6A). Also, the transpiration rate of alfalfa cultivars showed the same mode of action to stomatal conductance (Figure 6B). All of these proved that drought stress had significant effects on stomatal properties and caused the reduction in transpiration rate and stomatal conductance. Taiz and Zeiger (2007) proposed that the decrease in stomatal conductance under osmotic stress may be due to ABA accumulation as a signal from roots and stomatal closure under the stress condition. These results were in agreement with our findings about β-glucosidase activity as a marker of free ABA levels which indicated that drought stress increased ABA levels and led to stomata closure. Similar to our findings, Liang *et al.* (2002) and Heschel and Riginos (2005) reported that drought stress reduced stomatal conductance and transpiration rate of wheat and *Impatiense capensis*, respectively.

As concluding remarks, our findings showed that phenolic compounds as antioxidant agents increased under water stress. The higher phenolics content of Yazdi cultivar was resulted from lower activities of PPO, or may be from the higher synthesis of phenolics in this cultivar than Gharayonjeh which was not investigated in this study, and is suggested for further investigation.

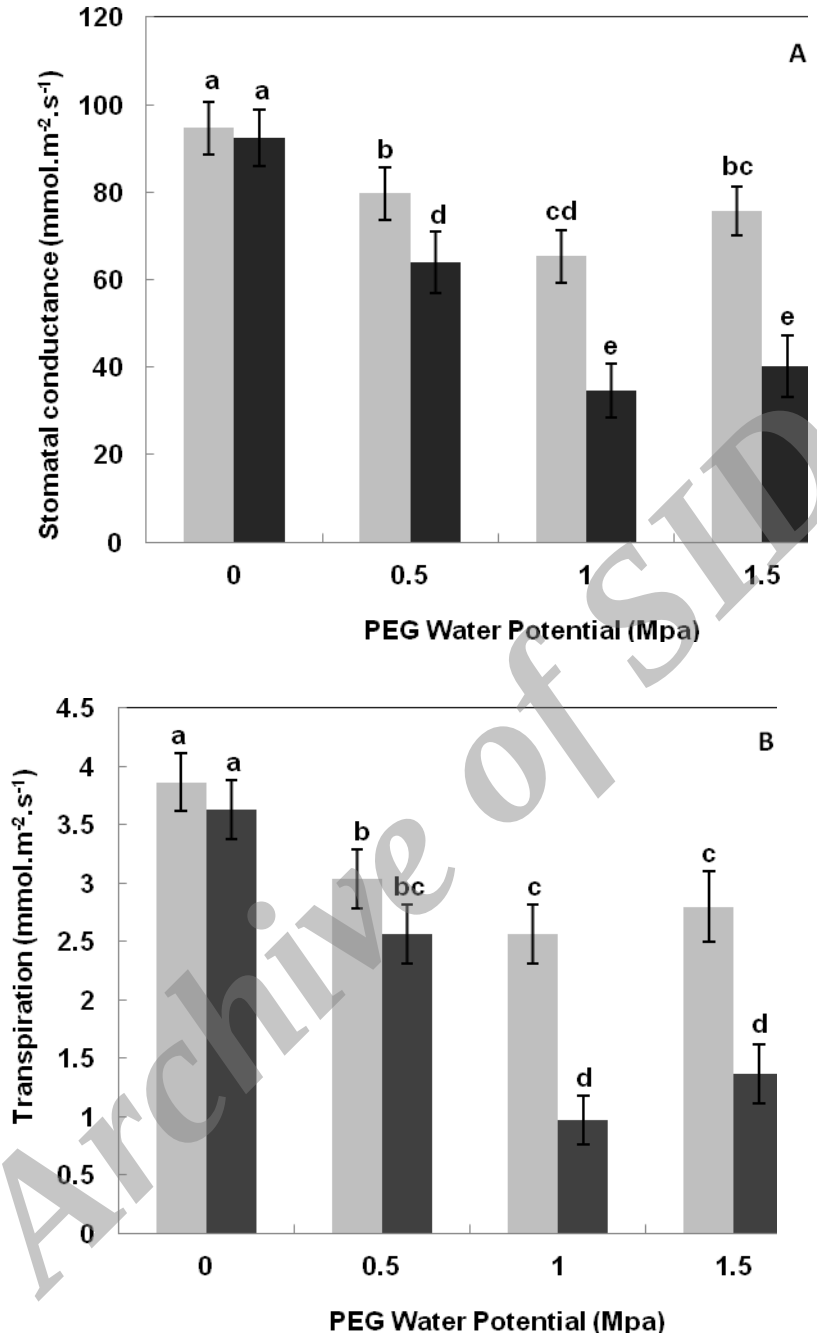

Figure 6. Stomatal conductance (A) and Transpiration rate (B) of Gharayonje (gray bars) and Yazdi (black bars) cultivars incubated on PEG 6000 solutions of 0, -0.5, -1.0 and -1.5 MPa water potential. Values are expressed as means of three independent replications ± standard error. Treatments with the same lower-case letters were not significantly different based on the mean comparison by Duncan's multiple range test at p≤ 0.05.

Also β-glucosidase which plays an important role in ABA metabolism showed higher activities in Yazdi cultivar. This result confirmed that Yazdi cultivar had better ability for stomata closure to encounter water stress.

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