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Antioxidant properties of two alfalfa (*Medicago sativa* L.) ecotypes in response to sodium chloride salinity stress

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Abstract
Biochemical and physiological responses of alfalfa under salinity stress were comparatively studied in a factorial experiment based on randomized complete block design by using Yazdi as the tolerant and Diabolourde as the sensitive ecotypes. Salt levels of 100, 150 and 200 mM were prepared by adding sodium chloride to the Hoagland half-strength culture medium. Total phenolics content, polyphenol oxidase (PPO), β-glucosidase and antiradical activities of leave′s extract, stomata properties and chlorophyll fluorescence parameters of the leaves including *Fv/Fm* and *Fv/Fo* were measured in response to salinity stress. Stomata characters were reduced in both ecotypes but chlorophyll fluorescence parameters only declined in Diabolourde but not in the Yazdi ecotype. The PPO and β-glucosidase activities increased in both ecotypes. The phenolics content and antiradical activities increased in the Yazdi ecotype at all salt levels but those of Diabolurde increased only at the higher salinity levels. Our observations indicated that the Yazdi ecotype manipulated biochemical and physiological responses more efficiently to alleviate the reduction of growth parameters under salinity.

Keywords: Alfalfa; β-glucosidase; Polyphenol oxidase; Stomata properties; Total phenolics

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Introduction

Soil salinity is one of the most important environmental stresses affecting crop yield in the world. High salinity severely reduces plant growth and productivity through ion toxicity, water deficit, or nutritional imbalance (Jamil *et al.* 2011). A consequence of soil salinity in plants is the production of reactive oxygen species (ROS). These molecules cause oxidative damage to cell components including membrane lipids, proteins and nucleic acids (Bartels and Sunkar 2005). To cope with oxidative stress, plants have developed different strategies such as producing antioxidant molecules and enzymes (Foyer and Noctor 2005). Antioxidant compounds sweep active oxygen species and avoid lipid peroxidation, cell damage and chlorophyll deterioration (Foyer *et al.* 2006).

Although the physiology of the salinity effect has been studied extensively in many plant species, the effect of this stress on polyphenol metabolism is not considerable (Popovic *et al.* 2016). Phenolic compounds can act as radical scavengers and lowers lipid autoxidation (Namiki 1990). These compounds are considered antioxidants that protect lipids from oxidative

damage. The synthesis and accumulation of polyphenols are provoked in response to salinity (Navarro *et al.* 2006; Valifard *et al.* 2014). Polyphenolic compounds take part in the defense against ROS, which is produced under environmental stresses through photosynthetic metabolism impairment (Sreenivasulu *et al.* 2000; Valifard *et al.* 2014).

Salt stress reduces cell expansion and division and consequently affects leaf size in plants (Fricke and Peters 2002; Curtis and Lauchli 1987; Abbruzzese *et al.* 2009). To diminish salt stress effects, plants can moderate their transpiration rate by control of stomata opening and change of leaf anatomy (Acosta-Motos *et al.* 2017; Abbruzzese *et al.* 2009). The control of stomata opening can control gas exchange and adjusts water use efficiency (Ceulemans and Mousseau 1994; Abbruzzese *et al.* 2009).

Alfalfa is the main forage species used as livestock food in Iran, due to its high quality, availability throughout the growing season and lower production costs. Because of the increasing human population and changing diet regimes, the demand for livestock products is growing. So, to provide more animal products, more forage is needed. Much of the cropland affected by salinity is located in the traditionally alfalfa-growing regions of the world (Johnson *et al.* 1992). Approximately, 25 million hectares of the surface area of Iran is affected by salinity (Khan *et al.* 2006). Because of the application of non-sustainable agriculture practices, soil salinity is increasing in Iran. Also, Iran is located in arid and semi-arid regions of the earth, therefore, osmotic stress is observed in large areas of farmlands.

Understanding the mechanisms of plant tolerance to salinity stress helps in developing salt-tolerant varieties. In this investigation, we studied some biochemical responses of sensitive and tolerant alfalfa ecotypes grown at different salinity levels.

Materials and Methods

Plant materials

Yazdi and Diabolourde have been selected previously as salt tolerant and salt-sensitive alfalfa ecotypes, respectively (Babakhani *et al.* 2011). Seeds of these ecotypes were provided by the Seed and Plant Improvement Institute, Karaj, Iran. The seeds were first surface sterilized with 5% sodium hypochlorite for 5 min and then washed three times with sterile water. The sterilized seeds were germinated in a growth chamber (Garouk, model GC400, Iran) at 24±4 °C, relative humidity of 70 ± 20% and dark condition for 5 days (Hosseini-Boldaji *et al*. 2012; Babakhani *et al.* 2017). Subsequently, seedlings were transferred to one-liter pots with half-strength Hoagland medium under 25±5 °C, illumination of PPFD of 400 µmol $m^{-2}s^{-1}$ prepared with fluorescent and incandescent lamps, 16/8 day/night photoperiod and aerated with an airflow of 400 ml min^{-1}. After 21 days, the plants were grown in nutrient solutions containing 0, 100, 150 and 200 mM NaCl for 14 days. The experiment was conducted in a house chamber with a day/night temperature of 27°C/18°C. After 14 days of salt treatment, the plants were harvested to be used for other assays (Babakhani *et al.* 2017).

Assay of total phenols

The concentration of total phenols in shoots was determined with Folin-Ciocalteu reagent following a colorimetric method (Singh *et al.* 2002). The amount of phenols was estimated based on a calibration curve obtained by gallic acid and expressed as milligrams of gallic acid equivalent per gram of dry weight (Delfanian *et al.* 2013).

DPPH· radical scavenging activity

The diphenylpicrylhydrazyl radical (DPPH·) scavenging activity was estimated based on Hanato *et al.* (1988). The DPPH· scavenging activity was measured as $(A_0 - A_1/A_0) \times 100$, where A_0 represents the absorbance of the control at 30 min and A_1 represents the absorbance of the sample at 30 min. IC_{50} ($\mu g.ml^{-1}$), the concentration of extract required to cause a 50% inhibition, was used to express the antiradical activity. Lower values of IC_{50} indicate a higher antioxidant activity of the extract (Msilini *et al.* 2012).

Polyphenol oxidase activity (EC 1.10.3.1)

Polyphenol oxidase (PPO) activity was assayed based on Kumar and Khan (1982) with slight modifications and expressed as $IU.g^{-1}DW$ (IU = a change of 0.1 absorbance per min).

Extraction and assay of ABA-β-D-glucosiadse

Extracellular fluid extraction was carried out according to Dietz *et al.* (2000). A modified procedure based on Garcia *et al.* (1993) was used to measure the extracellular β-glucosidase activity and expressed as IU. $g\ DW^{-1}$

Physiological characteristics

A Li 6200 portable photosynthesis system was utilized to measure the stomata conductance and transpiration rates of three leaves from each treatment around 12:00 AM, which were expressed as $mmol.m^{-2}.s^{-1}$.

Chlorophyll fluorescence parameters

The chlorophyll fluorescence parameters were determined according to Han *et al.* (2009) using a plant stress meter (Handy PEA V1.3, U.K.). These parameters were as follows:

Fv/Fm= Maximum quantum yield of PSII photochemistry.

Fv/Fo= Ratio of the variable fluorescence to the ground fluorescence.

Statistical analysis

The experiment was carried out as factorial based on the completely randomized design with three replications. After analysis of variance, the means were compared by Duncan's multiple range ($p \leq 0.05$). All statistical analyses were done by SAS software (version 9.2, SAS Institute) and the graphs were drawn by Excel 2007.

Results and Discussion

Effects of salinity on phenolics content

Our findings revealed that phenolic content of Yazdi ecotype increased significantly ($p \leq 0.001$) under different salinity levels as compared with the control, whereas it was only increased at 150 mM salinity in Diabolourde (Figure 1). Phenolic compounds content in plants is affected intensively by environmental factors (Ayaz *et al.* 2000; Bettaieb *et al.* 2007). In addition, salinity-

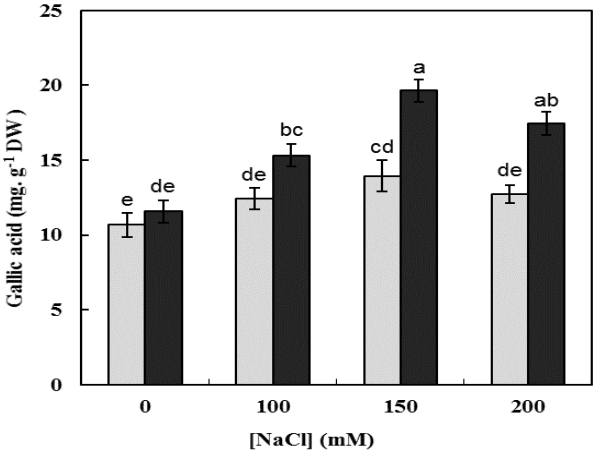

Figure 1. Changes in the total phenolic content of Diabolourde (grey bars) and Yazdi (black bars) ecotypes subjected to 0, 100, 150 and 200 mM NaCl salinity stress. Values are expressed as means of three replications ± standard error. Treatments with the same letters are not significantly different based on Duncan's test at $p \leq 0.05$.

induced metabolic process leads to an increase in the phenolic compounds (Ayaz *et al.* 2000). Phenolic compounds not only have antioxidant properties but also have high hydrophilicity due to hydroxyl and other hydrophilic groups (Song *et al.* 2000). This means that phenolic compounds could hold internal water in plants and reduce water loss under drought conditions (Chunlong *et al.* 2008). Our results imply that alfalfa ecotypes accumulated phenolic compounds to reduce oxidative damages caused by salinity and to decrease water loss from the leaves. The more tolerant ecotype had a greater ability to accumulate phenolic compounds in leaves than the sensitive ecotype.

Effects of salinity on PPO activity

The effect of salinity on PPO activity is presented in Figure 2. Sodium chloride increased the PPO activity in both ecotypes. Although the enzyme activity was similar in the control treatment of both sensitive and tolerant ecotypes, the rate of increase in the enzyme activity by increasing the salinity level in Diabolourde was higher than the Yazdi ecotype and the highest activity was recorded at 150 mM NaCl in Diabolourde. The reactive oxygen species increase when plants are exposed to various stress conditions. In this condition, ROS scavenging is important for plants to survive under these stresses (Jaleel *et al.* 2008; Michałowicz *et al.* 2009). The PPO activity has been proposed as a defense mechanism against stress conditions. It has been suggested that phenol oxidation by PPO leads to peroxide production, so peroxidase (POD) activity rises during the oxidation of phenolic compounds. In the previous study (Babakhani *et al.* 2001) the activities of peroxide scavenger enzymes including POD, ascorbate peroxidase (APX) and catalase (CAT) increased in both Yazdi and Diabolourde in response to salt stress. However, in the present study, PPO showed higher activity in Diaboloude than Yazdi. It is believed that PPO activity can alleviate oxidative damages of various

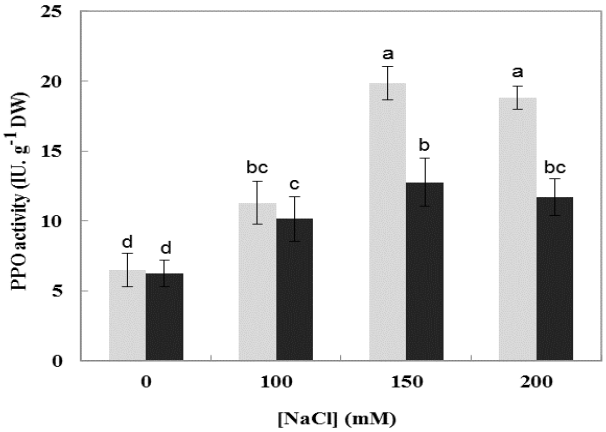

Figure 2. Changes in the polyphenol oxidase (PPO) activity of Diabolourde (grey bars) and Yazdi (black bars) ecotypes subjected to 0, 100, 150 and 200 mM NaCl salinity stress. Values are expressed as means of three replications ± standard error. Treatments with the same letters are not significantly different based on Duncan's test at $p \leq 0.05$.

stresses along with APX, POD and CAT (Niknam *et al.* 2006). Interestingly, the phenolics content of Diabolourde was lower than Yazdi, although according to Babakhani *et al.* (2011) the peroxide decomposing enzymes activities were higher in Yazdi than Diabolourde. The increase in PPO activity under salt stress has also been reported in barley (Ahmed *et al.* 2015).

Effects of salinity on antioxidant strength of the leaf extract

The change in IC_{50} as the marker of antioxidant strength of alfalfa extracts in response to salt stress is shown in Figure 3. The IC_{50} of both ecotypes decreased under salinity stress. This means that the antioxidant effects of extracts enhanced in both ecotypes under salinity stress as

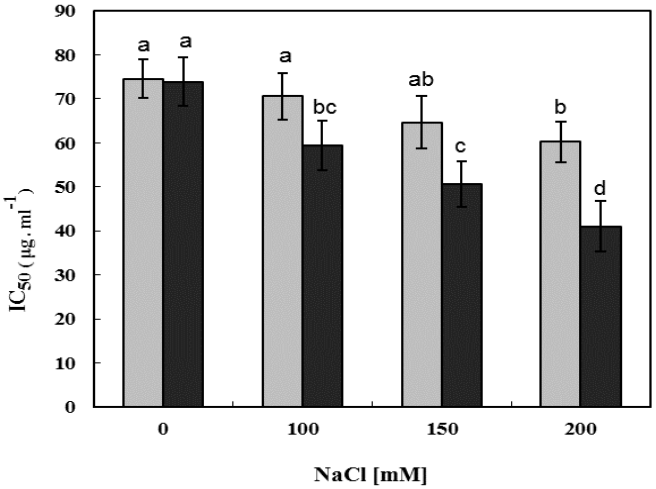

Figure 3. Changes in the DPPH free radical scavenging activity of Diabolourde (grey bars) and Yazdi (black bars) leaves extracts, subjected to 0, 100, 150 and 200 mM NaCl salinity stress. Values are expressed as means of three replications ± standard error. Treatments with the same letters are not significantly different based on Duncan's test at $p \leq 0.05$.

compared with the control. The rate of increase of antioxidant strength of extracts was higher in Yazdi than Diabolourde. The lowest IC_{50} was observed in the extract obtained from 200 mM NaCl in Yazdi. It has been indicated that phenolic compounds have an important role in scavenging free radicals (Falleh *et al.* 2011). Phenolic compounds are ideal for free radical scavenging activities because they possess: (1) phenolic hydroxyl groups to donate a hydrogen atom or an electron to a free radical, (2) extended conjugated aromatic system to delocalize an unpaired electron and (3) the ability to chelate transition metal ions (Dziedzic and Hudson, 1983; Dai and Mumper, 2010).

The phenolic compounds of the alfalfa ecotypes in this study were strongly related to their antioxidant properties measured by DPPH• radical scavenging activity (Figure 4). Thus, the phenolics were the main contributors to the antioxidant properties of the alfalfa extracts. This finding also supports the results about total

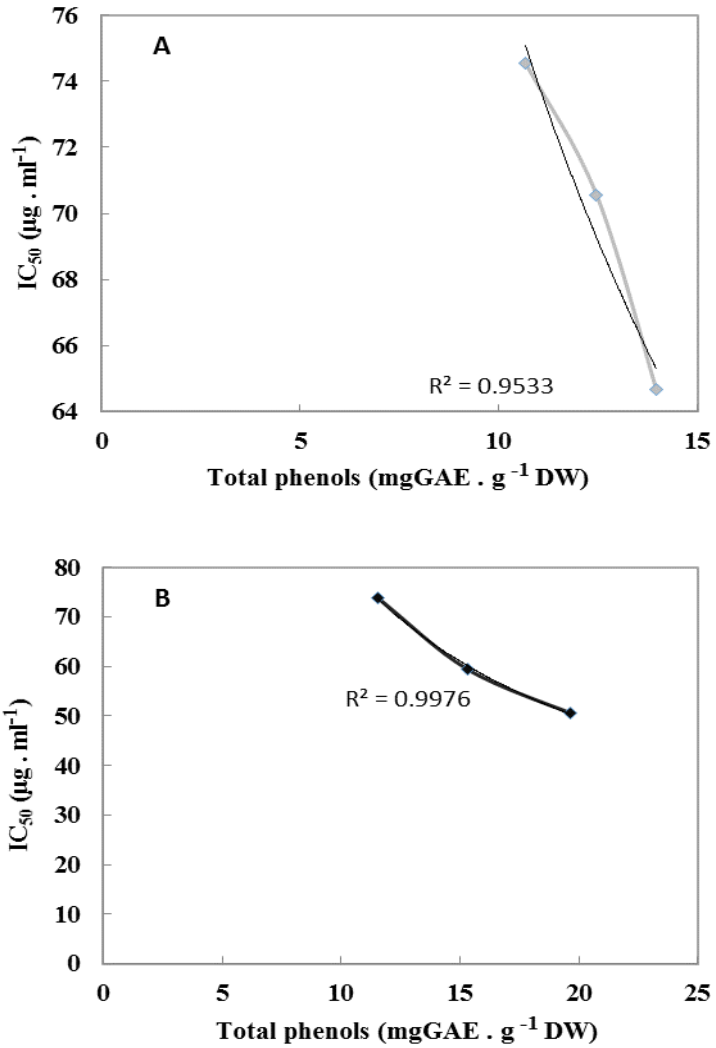

Figure 4. The relationship between DPPH scavenging activity and total phenolic content of Diabolourde (A) and Yazdi (B) extracts subjected to 0, 100 and 150 mM NaCl salinity stress. Values are expressed as means of three replications ± standard error. Treatments with the same letters are not significantly different based on Duncan's test at $p \leq 0.05$.

phenols content and PPO activity, which were higher in Yazdi and Diabolourde, respectively. Several authors have also reported a similar relationship between phenolic compounds and antioxidant activity (Proteggente *et al.* 2002; De-Beer *et al.* 2003). The increase in polyphenols content under increasing salinity has been reported in pepper (Navarro *et al.* 2006), mulberry (Agastian *et al.* 2000) and radish (Muthukumarasamy *et al.* 2000). The synthesis of phenolic compounds in response to salt stress has been reported in the tolerant and sensitive strawberry genotypes (Keutgen and Pawelzik 2008).

Effects of salinity on β-glucosidase activity

Salinity increased the β-glucosidase activity, as the indicator of free abscisic acid (ABA), at different salinity levels in both ecotypes (Figure 5) but the rate of increase was higher in Diabolourde at 150 and 200 mM salinity levels. ABA is a critical hormone for the adaptation of plants to various stresses (Kwang *et al.* 2006). The β-glucosidase hydrolyzes inactive ABA-GE to produce active free ABA (Kwang *et al.* 2006). Plants activate the inactive ABA by β-glucosidase to rapidly adjust ABA and respond to the stress conditions (Francisco 2011). In the present study, β-glucosidase activity increased in both ecotypes to possibly increase cellular free ABA under salt stress. In earlier studies, it has been pointed out that β-glucosidase liberates ABA from ABA-GE under stress conditions (Kwang *et al.* 2006, Kato-Noguchi and Tanaka, 2008; Babakhani *et al.* 2017).

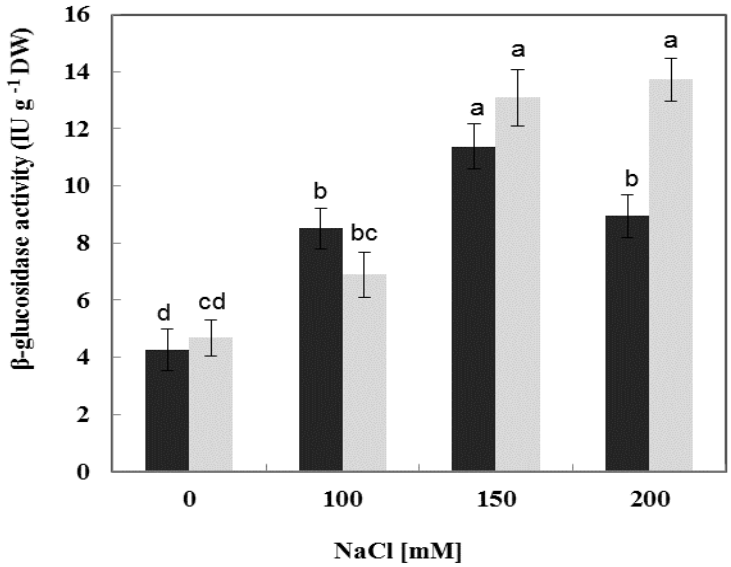

Figure 5. Changes in the β-glucosidase activity of Diabolourde (grey bars) and Yazdi (black bars) ecotypes subjected to 0, 100, 150 and 200 mM NaCl salinity stress. Values are expressed as means of three replications ± standard error. Treatments with the same letters are not significantly different based on Duncan's test at $p \leq 0.05$.

Effects of salinity on stomata properties

Effects of salt stress on stomata properties are shown in Figure 6. Under salinity conditions, stomata conductance (Figure 6A) and transpiration rate (Figure 6B) decreased in both ecotypes as compared to the control but the reduction in Diabolourde was higher than Yazdi at 150 and 200 mM NaCl. Therefore, the lowest stomata conductance and transpiration rate were observed in the Diabolourde ecotype at 200 mM NaCl. The same findings were reported by Vysotskaya *et al.* (2010) in barley under salinity.

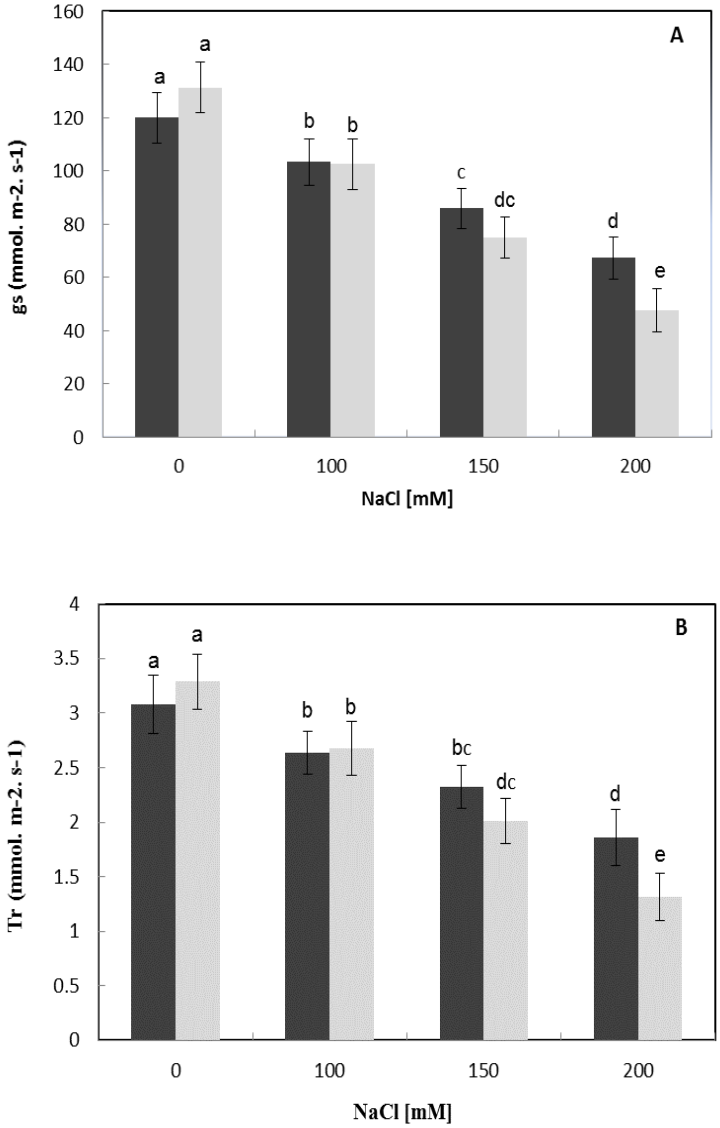

Figure 6. Changes in stomatal conductance (A) and transpiration rate (B) of Diabolourde (grey bars) and Yazdi (black bars) ecotypes subjected to 0, 100, 150 and 200 mM NaCl salinity stress. Values are expressed as means of three replications ± standard error. Treatments with the same letters are not significantly different based on Duncan's test at $p \leq 0.05$.

These findings indicate that salt stress has increased free ABA levels and has led to stomata closure.

Salt stress reduces root hydraulic conductivity which causes a decrease in water flow from roots to shoot (Rengasamy and Olsson 1993) and consequently, stomatal closure to maintain the water status (Robinson *et al.* 1997). Therefore, it could be assumed that the higher amounts of gs and Tr in the Yazdi ecotype can be attributed to its higher resistance to the reduction in root hydraulic conductivity as compared to Diabolourde under salinity stress.

Effect of salinity on chlorophyll fluorescence

The effect of salt stress on chlorophyll fluorescence parameters including Fv/Fm and Fv/Fo are shown in Figures 7A and 7B, respectively. Fv/Fm declined in Diabolourde under 200 mM salinity only, while that of Yazdi ecotype did not differ significantly. Fv/Fo declined in Diabolourde at all salinity levels but it was reduced only at 200 mM NaCl in the Yazdi ecotype. Fv/Fm declined by 3.9% and 15.1% under salinity level of 200 mM in Yazdi and Diabolourde ecotypes, respectively. The decline in Fv/Fo was 9.3% and 42.1% in Yazdi and Diabolourde ecotypes, respectively. Ghassemi-Golezani *et al.* (2011) also reported a reduction in chlorophyll fluorescence of soybean under salinity stress. At salt stress conditions, the decrease in the activity of the photosynthetic apparatus resulted in lower values of Fv/Fm and Fv/Fo in the Diaboloure ecotype of alfalfa as compared to the control. Under stress treatments, the stomata limitation on gas exchange was possibly concomitant with the reduction in the consumption rate of ATP and NADPH for CO_2 assimilation, which decreased the chloroplastic linear electron transport rate and then Fv/Fm (Baker and Rosenqvist 2004; Hura *et al.* 2007; Li *et al.* 2012). Also, the decrease in Fv/Fo at the stress conditions can be attributed to the water-splitting system at the donor part of PS II (Hura *et al.* 2007; Li *et al.* 2012). The same observations were reported in Arabidopsis and Thellungiella under salt stress (Stepien and Johnson 2009) and alfalfa under osmotic stress (Babakhani *et al.* 2017).

Conclusion

The Diabolourde ecotype showed higher activities of β-glucosidase under salt stress which hydrolyzed ABA-GE to free ABA. Stomata properties of both ecotypes were in concordance with the β-glucosidase activity. However, the reduction in chlorophyll fluorescence parameters was only observed in Diabolourde but not in the Yazdi ecotype. The generation of ROS under stress conditions may have caused injuries to membranes and consequently resulted in higher activities of PPO and lower phenolics and chlorophyll contents of Diabolourde as compared to the Yazdi ecotype. The results of this experiment suggested that the Yazdi ecotype was able to manipulate the biochemical and physiological responses more efficiently, which enabled this ecotype to reduce the negative effects of salinity on growth parameters.

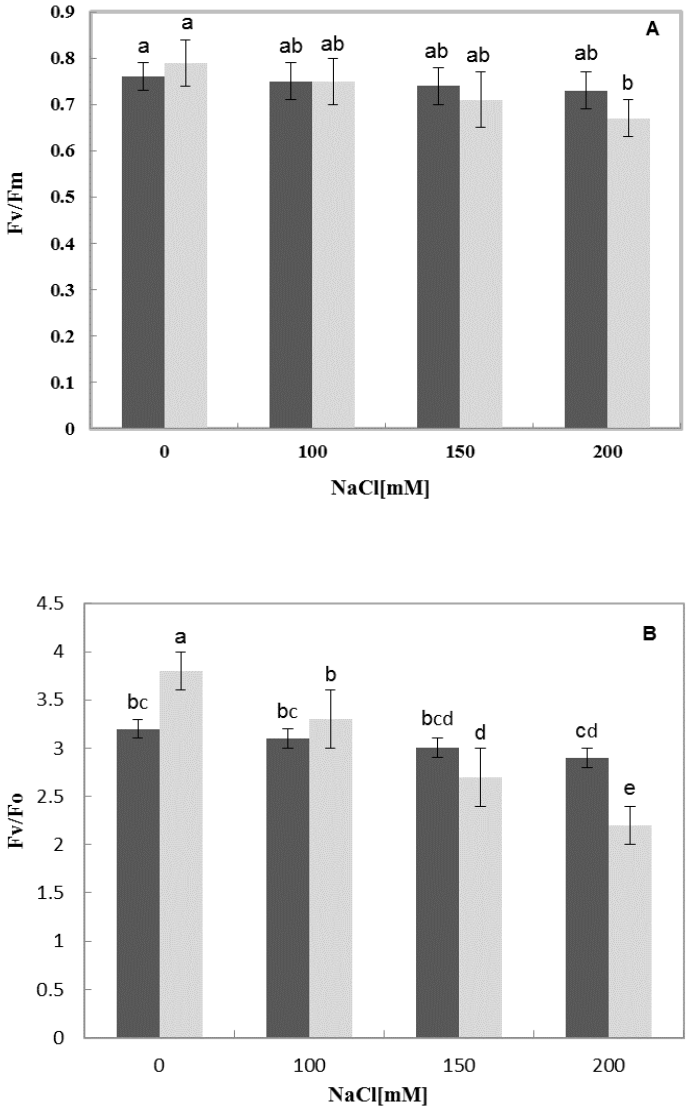

Figure 7. Changes in Fv/Fm (A) and Fv/Fo (B) of Diabolourde (grey bars) and Yazdi (black bars) ecotypes subjected to 0, 100, 150 and 200 mM NaCl salinity stress. Values are expressed as means of three replications ± standard error. Treatments with the same letters are not significantly different based on Duncan's test at $p \leq 0.05$.

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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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ویژگی‌های آنتی اکسیدانی دو اکوتیپ یونجه (*Medicago sativa* L.) در پاسخ به تنش شوری کلرید سدیم

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چکیده

پاسخ‌های بیوشیمیایی و فیزیولوژیک یونجه در یک آزمایش فاکتوریل بر پایه بلوک‌های کامل تصادفی با استفاده از اکوتیپ‌های یزدی و دیابلورده به ترتیب به عنوان اکوتیپ‌های مقاوم و حساس بررسی شد. سطوح شوری ۱۰۰، ۱۵۰ و ۲۰۰ میلی مولار با اضافه کردن نمک کلرید سدیم به محیط کشت نیم-قدرت هوگلند تهیه شد. اثر تنش شوری بر ویژگی‌های بیوشیمایی و فیزیولوژیک شامل محتوی فنلی کل، فعالیت آنزیم‌های پلی فنل اکسیداز و بتاگلوکوزیداز در عصاره برگی، رفتار روزنه‌ای و پارامترهای فلورسانس کلروفیل (F_v/F_0 و F_v/F_m) تعیین شد. نتایج نشان داد که ویژگی‌های روزنه‌ای در هر دو اکوتیپ کاهش یافت ولی فلورسانس کلروفیل فقط در دیابلورده کاهش نشان داد و در اکوتیپ یزدی تغییری مشاهده نشد. میزان کاهش این پارامترها بین دو اکوتیپ متفاوت بود. فعالیت آنزیم‌های پلی فنل اکسیداز و بتاگلوکوزیداز در هر دو اکوتیپ افزایش یافت. محتوی فنلی کل و فعالیت آنتی اکیسیدانی در تمامی تیمارها در اکوتیپ یزدی افزایش نشان داد در حالی که در اکوتیپ دیابلورده تنها در تیمارهای بالای شوری افزایش یافت. میزان افزایش این پارامترها در دو اکوتیپ با یکدیگر متفاوت بود. این مشاهدات نشان می‌دهد که اکوتیپ یزدی از پاسخ‌های بیوشیمیایی و فیزیولوژیک با کارایی بالاتر برخوردار بود تا اثرات کاهشی شوری بر پارامترهای رشد را کم کند.

واژه‌های کلیدی: بتاگلوکوزیداز؛ پلی فنل اکسیداز؛ محتوی فنل تام؛ ویژگی‌های روزنه‌ها؛ یونجه