The investigation of some biochemical and physiological responses of alfalfa (*Medicago sativa L.*) cultivars from Iran to NaCl salinity stress

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Abstract

In order to investigate the effects of salt stress on biochemical and physiological responses of two cultivars of alfalfa (Medicago sativa L.) namely, Diabolourde and Yazdi, chlorophyll content, growth parameters, and proline contents of roots and shoots, reducing sugars contents of roots and shoots, and membrane injuries of the plant samples were subjected to 0, 100, 150, and 200 mM NaCl treatment in a factorial experiment based on a completely randomized block design. Findings showed that chlorophyll content, root dry weight, and shoot dry weight decreased in both cultivars under salt stress ($p \leq 0.05$). The reduction rate was more severe in Diabolourde cultivar than Yazdi. The root to shoot ratio had no significant differences in Diabolourde while increased significantly in Yazdi under 150 and 200 mM NaCl in comparison with control ($p \leq 0.05$). Reducing sugars content and proline content of roots and shoots increased significantly in both cultivars. The enhancing rates were more severe in Yazdi than Diabolourdeh. Also, the electrolyte leakage as a marker of membrane injuries increased in both cultivars as the enhancing rate was higher in Diabolourde cultivar in comparison with Yazdi. These findings showed that Yazdi cultivar employed resistance mechanisms more effectively than Diabolourde and therefore, it suffered lower injuries; higher growth parameters and lower membrane leakage in Yazdi cultivar are in agreement with this claim. According to the findings, Yazdi cultivar is proposed as a more tolerant variety for cultivation in saline area.

Keywords: Alfalfa cultivars; growth parameters; proline; reducing sugars; salinity

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Introduction

The quantity and quality of irrigation water are the main limiting factors to the extension of the agriculture in the arid and semi-arid regions of the world. The saline area has still been increasing as a result of improper irrigation water management (Abdel Latef and Chaoxing, 2014). Salinity is one of the most limiting factors for agricultural productivity worldwide causing the decrease in crop average yields by more than 50% (Singh et al., 2015).

Photosynthesis and cell growth are among the primary processes which are affected by

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salinity (Chaves et al., 2009). In addition to the toxic effects of the sodium and chloride ions, salinity disturbs the plant's water relations due to the decreased availability of water from soil solution as a result of lowered osmotic potential. Different plant species have developed various mechanisms to cope with these effects (Hameed et al., 2012). Physiological changes including stomatal conductance, lowered water potential, and osmotic adjustment, in plants growing under salt conditions have been developed as effective responses to saline condition (Alvarez and Sanchez-Blanco, 2014).

Abiotic stresses which affect plant growth lead to the overproduction of reactive oxygen species (ROS) which are highly reactive and toxic. ROS comprise both free radicals (O_2-, •OH, HO_2• and R-O•), and non-radical molecular forms (H_2O_2 and $1O_2$). The excess of ROS causes damage to proteins, lipids, carbohydrates, and DNA which ultimately results in the disruption of cellular homeostasis and subsequently cellular death (Swapnil et al., 2017). Thus, to maintain growth and productivity under stress conditions, plants have to activate several strategies to scavenge the enhanced generation of ROS. Plants have developed different strategies involving antioxidant molecules and enzymes that protect them against the potentially cytotoxic species of activated oxygen and to cope with the mentioned challenges (Abogadallah et al., 2010).

Stress tolerance/adaptation seems to be correlated with stimulation of antioxidant mechanisms and the enhanced ability to remove ROS (Bettaieb et al., 2007). It is important to produce salt tolerant plants to increase their adaptability to grow in salty lands. Therefore, understanding the mechanisms of plant tolerance to high salinity is a crucial environmental research topic (Gupta and Huang, 2014).

Alfalfa is the oldest and the most important forage crop in the world and currently is cultivated as a nitrogen source and soil-conserving perennial crop in low-input agricultural systems (Naseri and Marefat, 2008). Alfalfa is moderately tolerant to salinity but there is high variation between cultivars of alfalfa; therefore, selection among the germplasms should lead to increasing salt tolerance (Bertrand et al., 2015). In the current study, the differences in some biochemical responses of two alfalfa cultivars were investigated in order to discriminate protective mechanisms in more tolerant cultivar.

Material and Methods

Plant material

On the basis of a previous study (Babakhani et al., 2011), Yazdi and Diabolourde were selected as alfalfa salt tolerant and sensitive cultivars, respectively. 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). Subsequently, seedlings were transplanted to 1L pots containing half strength Hoagland solution and were grown under controlled condition at 25±5 °C, illumination of PPFD of 400 µM $m^{-2}s^{-1}$ prepared with combined fluorescent and incandescent lamps with 16/8 day/night photoperiod and aerated with an air flow of 400 ml min^{-1} for 15 days. The pH of the solutions was adjusted to 7.0 to 7.5 daily, using 200 mM KOH or HCl. The plants were grown for 21 days in controlled condition followed by another 14 days in the presence of 0, 100, 150, and 200 mM NaCl added to the nutrient solutions. Experiments were conducted in a house chamber with the average temperature of 27/18 °C day/night. The plants were harvested at the end of the 14th day of salt treatment for later experiments.

Growth parameters

For determination of dry weight, samples were divided into root and shoot fractions and oven dried at 70 °C for 48 h and afterward weighed. Shoot and root dry weights were expressed as g. $plant^{-1}$.

Chlorophyll determination

The chlorophyll was extracted by homogenizing 200 mg fresh weight of leaves in 10 ml acetone 80% solution. After centrifugation for 10 min at 4000 × g, Chlorophyll content was

determined spectrophotometrically in the supernatant at 646.6 and 663.6 nm as described by Porra (2002) using the following equation:

Total Chl (µg.ml-1) = 17.76 (A646.6) + 7.34 (A663.6).

Extraction and determination of reducing sugars

Sugars extraction was carried out following the method of Naureen and Naqvi (2010) with some modifications. Plant materials (roots or shoots, 250 mg) were collected at 10 to 11 AM then frozen in liquid nitrogen and subjected to a triple extraction of ethanol-soluble sugars (ESS) by boiling in ethanol 80% at final volume of 20 ml in a water bath. Five ml of chloroform were added to the extract and mixed by vortex, then centrifuged at 12000 × g for 3 min. The supernatant was used to determine the reducing sugars as described by Nelson (1944). The concentration of sugars was expressed as $\mu g.g^{-1}$ DW.

Proline assay

Proline content was determined according to the modified method of Bates et al. (1973). Briefly, two hundred milligrams of frozen plant materials (roots or shoots) were homogenized in 1.5 ml of 3% sulfosalicylic acid. The mixture was then centrifuged at 13000 × g for 10 min. Half milliliter of the supernatant was then added into a test tube and 1 ml of glacial acetic acid and 1 ml of freshly prepared acid ninhydrin solution were added to the tube. The tubes were incubated in a water bath for 1 h at 100 °C and then allowed to cool to the room temperature. Two milliliters of toluene were added and mixed on a vortex mixer for 20 min. The test tubes were left for the separation of toluene and aqueous phase. The toluene phase was carefully driven out into a glass test tube and the absorbance was measured at 520 nm in a spectrophotometer. The concentration of proline was calculated from a proline standard curve and was expressed as $\mu g.g^{-1}$ DW.

Electrolyte leakage

Leaf membrane damage was determined by recording of electrolyte leakage (EL) as described by Valentovic et al. (2006) with a few modifications. Plant leaves (0.3 g) were washed with deionized water and were placed in tubes with 20 ml of deionized water and incubated for 24 h at 25 °C. Subsequently, the electrical conductivity of the solution (EC1) was measured by conductivity meter (ME977-C, India). Samples were autoclaved at 120 °C for 20 min and then were cooled. Final conductivity (EC2) was measured after equilibration at 25 °C. The EL was defined as:

EL (%) = (EC1/EC2) × 100.

Statistical Analysis

The experiments were performed in a factorial experiment based on a completely randomized design. The data are shown as the mean ± standard error (S.E.) of four replicates (for experiments of proline, reducing sugars and chlorophyll contents, and growth parameters determination) or three replicates (for experiment of electrolyte leakage). Statistical analysis of data was performed by two-way analysis of variance (ANOVA) method. The mean comparison performed using post-hoc Duncan's multiple range test to discriminate significance (defined as $p<0.05$). The statistical analysis was carried out using the statistical analysis system software (version 9.2, SAS institute); graphs were drawn by Excel 2007.

Results

To investigate the physiological and biochemical responses of two alfalfa cultivars under salt stress, all experiments were evaluated 14 days after stress exposure. Under normal conditions, the more tolerant cultivar showed lower levels of chlorophyll than the sensitive one (Table 1). However, the chlorophyll content of both cultivars was reduced significantly under all saline treatments ($p\leq0.001$, Table2). Interestingly, the decreasing rate of the sensitive cultivar was more severe than the tolerant one, as chlorophyll

Table 1
Mean comparison of the effect of salt stress on some biochemical and physiological parameters of Yazdi and Diabolourde cultivars of alfalfa; data are means ± SE of 4 separate replicates.

Parameters	Cultivar	Salt concentration (mM)			
		0	100	150	200
Chlorophyll content	Yazdi	39.7±2.8 b	21.5±2.1 c	20±1.4 cd	14.6±2.1 e
	Diabolourde	46.3±3.4 a	22.8±2.2 c	17.9±1.6 de	8.86±1.29 f
Shoot dry weight	Yazdi	0.081±0.003 a	0.065±0.003 b	0.053±0.003 c	0.051±0.003 c
	Diabolourde	0.077±0.003 a	0.051±0.002 c	0.039±0.003 d	0.028±0.002 e
Root dry weight	Yazdi	0.051±0.003 a	0.044±0.003 b	0.042±0.002 b	0.042±0.002 b
	Diabolourde	0.05±0.002 a	0.032±0.001 c	0.023±0.002 d	0.017±0.002 e
Root to Shoot ratio	Yazdi	0.62±0.05 b	0.67±0.04 b	0.79±0.06 a	0.83±0.05 a
	Diabolourde	0.65±0.02 b	0.62±0.05 b	0.59±0.06 b	0.58±0.06 b
Shoot proline	Yazdi	3.18±0.75 fe	5.98±0.96 d	13.1±1.4 b	19.2±1.2 a
	Diabolourde	2.53±0.65 f	4.32±0.82 e	8.33±1.07 c	14.4±1.26 b
Root proline	Yazdi	2.48±0.51 fe	4.85±0.71 d	8.7±0.81 b	13±1.23 a
	Diabolourde	1.88±0.54 f	3.24±0.48 e	6.59±0.8 c	8.91±0.81 b
Shoot reducing sugars	Yazdi	127.4±11.7 de	150.3±11.3 bc	161.9±9.4 ab	173.4±11.4 a
	Diabolourde	114.3±8.4 e	125.8±9.1 de	135±6.3 d	137.7±9.7 cd
Root reducing sugars	Yazdi	19.7±1.4 e	32.5±1.7 b	33.6±1.7 b	39±1.6 a
	Diabolourde	17.1±1.2 f	20.2±1.4 de	22.2±1.4 d	28.3±1.5 c
Electrolyte leakage	Yazdi	13.6±1.6 e	22.1±2.1 d	26.1±2.1 dc	28.9±2 bc
	Diabolourde	14.8±2 e	22.8±1.6 dc	32.7±2 b	40±1.9 a

Mean values with different letters are significantly different according to Duncan's multiple range test ($p \leq 0.05$).

content of Yazdi was higher than Diabolourde under 200 mM treatment.

Growth parameters of alfalfa including shoot and root dry weights and root to shoot ratio under salt stress are shown in Table 1. According to the results, root and shoot weights reduced under stress condition in both cultivars as compared with control (Table 2). The decline in root and shoot weights of Diabolourde were more severe than that of Yazdis.

The root to shoot ratio of alfalfa cultivars under various salinity levels is shown in Table 1. According to our findings, since root and shoot dry matters decreased in both cultivars under salinity treatments, this parameter increased in Yazdi cultivar in 150 and 200 mM treatments in comparison with the control ($p \leq 0.05$) while in Diabolourde it was not affected significantly.

Salinity effects on proline and reducing sugars contents of alfalfa cultivars as osmotic adjustment responses are shown in Table 1. According to our results, proline contents of roots and shoots were increased in both Yazdi and Diabolourde under all salinity levels in comparison with the control (Table 2). In response to salinity, the augmentation of proline in both roots and shoots were more acute in Yazdi than in Diabolourde.

In the same way, the reducing sugars contents of roots and shoots increased in both Yazdi and Diabolourde cultivars under various salinity levels (Table 2) although the enhancing rate of sugars content was faster in Yazdi compared with Diabolourde.

The observations on electrolyte leakage indicated that the destruction of cell membranes of both cultivars was enhanced as the NaCl concentration of medium increased (Table 1). The electrolyte leakage of Diabolourde was more severe than that of Yazdi. The highest electrolyte leakage was observed in Diabolourde cultivar under 150 and 200 mM treatments.

Discussion

According to Noreen and Ashraf (2009), the decrease in chlorophyll content might have been due to salt-induced increase in the activity of chlorophylase (the chlorophyll degrading enzyme). Also, antioxidant enzymes prevent degradation of leaf chlorophyll. The antioxidant enzymes activities of both cultivars were investigated in a previous study (Babakhani et al., 2011) and the findings are in agreement with the present study. In accordance with our findings, the decrease in chlorophyll content under salinity

Table2: The analysis of variance of salinity effects on physiological and biochemical responses of alfalfa cultivars (experiments with 4 replicates).

Source		Root sugar			Shoot sugar			Root proline		
	df	MS	F	P	MS	F	P	MS	F	P
Cultivars	1	684.8	288.4	0.0001	5051	51.9	0.0001	35.5	59.2	0.0001
Salt	3	317.6	133.7	0.0001	1830	18.8	0.0001	121.6	202.8	0.0001
Species* Cultivars	3	40.6	17.1	0.0001	177.1	1.82	0.1702	4.37	7.29	0.0012
Error	24									
Total	31									

Source		Shoot proline			Shoot dry weight			Root dry weight		
	df	MS	F	P	MS	F	P	MS	F	P
Cultivars	1	70.3	57.6	0.0001	0.0015	142.6	0.0001	0.0016	212.7	0.0001
Salt	3	310.7	254.8	0.0001	0.0023	224.7	0.0001	0.0006	90.3	0.0001
Species* Cultivars	3	9.06	7.43	0.0011	0.0001	9.65	0.0002	0.0002	30.9	0.0001
Error	24									
Total	31									

Source	df	Root to shoot ratio			Chlorophyll content			Source	df	Electrolyte leakage		
		MS	F	P	MS	F	P			MS	F	P
Cultivars	1	0.106	33.5	0.0001	0.009	0.00	0.9665	Cultivars	1	153	13.14	0.0023
Salt	3	0.008	2.66	0.0707	1445	282.9	0.0001	Salt	3	475.6	40.85	0.0001
Species* Cultivars	3	0.033	10.5	0.0001	55.4	10.8	0.0001	Species* Cultivars	3	40.55	3.48	0.0406
Error	24							Error	16			
Total	31							Total	23			

stress was higher in salt sensitive wheat cultivars compared to the more tolerant cultivar (Khan et al., 2009). The decrease in chlorophyll content under salinity conditions was also reported by Nxele et al. (2017) and Nazarbeygi et al. (2011).

It has been suggested that stomata closure upon salt stress may limit the entry of CO_2. The decrease in plant growth may be resulted from reduced CO_2 assimilation which disturbs photosynthesis, declined turgor of expanding tissues, and insufficient osmoregulation (Brodribb and Mc-Adam, 2011). Also, the reduction in plant weight under salinity stress has been explained by the stop in cell division and cell elongation caused by the decrease of water potential in plant cells. Similar to chlorophyll contents, reductions in root and shoot dry weights were more intensive in Diabolurde in comparison with Yazdi cultivar. Our results are in agreement with those of Kurum et al. (2013) in pumpkin varieties and Wang et al. (2009) in alfalfa varieties.

According to Iqbal and Ashraf (2013), the changes in partitioning under osmotic stress were often in favor of root growth which were reflected in increased root to shoot ratios. This also may be due to a faster osmotic adjustment and a slower turgor loss in roots than in shoots (Kroeger et al., 2011). The increase in root to shoot ratios of Yazdi cultivar indicated that roots became stronger sinks than the shoots and plants invested more assimilates in root growth compared with shoot under salinity stress.

The loss of intracellular water is a major consequence of NaCl stress; therefore, plants accumulate various metabolites that are known as compatible solutes to prevent water loss from the cell and protect the cellular proteins (Harishchandra et al., 2010). Frequently observed metabolites with an osmolyte function are sugars mainly fructose and sucrose, charged metabolites like glycine-betaine, and proline. Under stress conditions the accumulation of these osmolytes can lower water potential inside the cell which helps plants to uptake water from the environment (Nxele et al. 2017). The function of compatible solutes is not restricted to osmotic adjustment only. These compounds may also protect plants against damage by scavenging reactive oxygen species (Singh et al., 2015). Our results indicated that proline and reducing sugars act as osmotic adjusting agents in alfalfa cultivars. The more tolerant cultivar has greater ability for osmolyte accumulation compared with its sensitive counterpart. Same results for proline accumulation under salt stress condition were reported by Nxele et al. (2017) in sorghum and

Yousfi et al. (2010) in Medicago species. Sugars accumulation under noted condition also reported in Schenkia spicata (Misic et al., 2012) and Medicago species (Yousfi et al., 2010).

Under environmental stresses plant membranes are subject to changes often associated with enhances in permeability and lack of integrity (Taibi et al., 2016). Therefore, the ability of a cell membrane to control the rate of ion traffic in and out of cells is used as a test of membrane damage. Reactive oxygen species (ROS) are regarded as the main source of damage to cells under biotic and abiotic stresses (Nxele et al., 2017). Evidences suggest that membranes are the primary sites of salinity injury to cells and organelles because ROS can react with unsaturated fatty acids to cause peroxidation of essential membrane lipids in plasma membrane or intracellular organelles (Nxele et al., 2017). Peroxidation of plasma membrane lipids leads to the leakage of cellular contents. According to a previous study (Babakhani et al., 2011) and our results, antioxidant enzymes worked in lower efficiency manner in Diabolourde as compared with Yazdi cultivar; therefore, ROS injuries which are determined as electrolyte leakage, were more severe in Diabolourde than in Yazdi. The same results were reported by Kaya et al. (2013) in rice and Bayat et al. (2012) in calendula under salt stress condition.

Conclusion

As a concluding remark, higher proline and reducing sugars content of roots and shoots, growth parameters, and lower membrane leakage in Yazdi cultivar in the present study show that Yazdi cultivar employed resistance mechanisms more effectively than Diabolourde, and suffered lower injuries. Therefore, Yazdi as a more tolerant cultivar is suggested for cultivation in saline area.

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