

TRIADIMEFON AMELIORATES NEGATIVE EFFECTS OF *IN VITRO* SALT STRESS ON TWO CULTIVARS OF ALFALFA (*Medicago sativa* L.)

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ABSTRACT

Triadimefon (TRD) is a member of triazole plant growth regulator group which is responsible for increasing stress tolerance. Effects of TRD on two cultivars of *Medicago sativa* (Hamedani and Yazdi) were investigated. The *in vitro* grown plantlets in MS medium were cultured in TRD at concentration 1.0, 2.0 and 4.0 mg/l combined with 0, 100 and 140 mM NaCl respectively. The changes in growth parameters, photosynthetic pigment, carbohydrate, α -tocopherol, glycine betain, proline and proline dehydrogenase (PDH) activity as well as its expression were measured after four weeks post treatment. NaCl treatment decreased overall plant growth and reduced the total chlorophyll, carbohydrate content, PDH activity and increased α -tocopherol, glycine betain content and proline content. The level of PDH transcript was changed in different combinations of NaCl and TRD, however, at 140 mM NaCl and 2 mg/l TRD expression was decreased. When plants were subjected to salt stress, TRD minimized the inhibitory effects of NaCl on plant growth by increasing, total chlorophyll, carbohydrate, α -tocopherol, glycine betaine content.

Key words: Alfalfa, carbohydrate, salt stress, triadimefon

INTRODUCTION

Triazole compounds have both fungicidal and plant growth regulator (PGR) properties. Its also protect the plants against several types of abiotic stresses. The PGR properties of triazoles are mediated through their interference with the isoprenoid pathway and the consequent alterations in the important plant biochemical balances including gibberellin, abscisic acid (ABA) and cytokinins. Triazoles inhibit cytochrome P450 mediated oxidative demethylation reactions required for the synthesis of ergosterol. It also essential in the process of converting kaurene to kaurenoic acid in the gibberellin biosynthetic pathway that affect the isoprenoid pathway altering certain plant hormonal levels through inhibiting gibberellin synthesis, reducing ethylene evolution and increasing cytokinin levels (Fletcher *et al.*, 2000). Triazole compounds influence hormonal balance, photosynthetic rate, enzyme activities and lipid peroxidation ending up with different components in various crop plants (Jaleel *et al.*, 2007). Triadimefon [1-(4-chlorophenoxy)-3,3-dimethyl-1-

(1H-1,2,4-triazole-1-yl)-2-butanone] is a triazole derivative compound with PGR properties (Fletcher *et al.*, 2000; Jaleel *et al.*, 2007).

Salinity is one of the major abiotic stresses affecting development, growth and production efficiency of the plants. These restrictive effects of salinity are due to osmotic inhibition, ionic toxicity and disturbance in the uptake and translocation of nutritional ions leading to physiological and biochemical disturbances ending with death of the plant cells (Xiong and Zhu, 2002).

In plant, the accumulation of metabolites acting as compatible solutes is a possible universal response to changes caused by external stressful stimuli. Metabolites affecting osmosis like sugar alcohols, complex sugars and charged compounds are reported in plants under unfavorable conditions (Sotiropoulos, 2007). Proline and glycine betaine are known to beneficial as compatible osmolytes that either protect macromolecules or serve as scavengers of ROS in stressful conditions (Ashraf and Foolad, 2007).

Degradation of proline is regulated during development and stress via conversion of proline to glutamate using proline dehydrogenase (PDH). Salt stress decreased the PDH activity in potato

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(Rahnama and Ebrahimzadeh, 2004) and *Brassica juncea*, (Madan *et al.*, 1995) but increases the level of proline. Salinity also affects the gene expression, protein biosynthesis and protein modification (Parvaiz and Satyawati, 2008). Alteration in protein structure is vital as plants are either in conditions to response to stressful environment or if they are getting adapted to environmental conditions (Vierstra, 1993).

Considerable attention has been developed to induce stress tolerance in plants. The approaches are include genetic engineering (McKersie *et al.*, 1988), breeding (Vettakkorumakankav *et al.*, 1999), *in vitro* selection, and the use of growth regulators (Senaratna *et al.*, 2000, Baninasab and Ghobadi, 2011). Exogenous PGR has been reported to reduce the adverse effect of salinity on growth and production of some crops (Sankar *et al.*, 2006). Alfalfa, a leguminous plant often encounters significant biomass reduction due to severe salt stress. Interestingly, different alfalfa cultivars respond differently to salt stress (Wang and Han, 2009). To our knowledge, no reports regarding the effects of exogenous TRD on alfalfa under salt stress has been published. Thus, the objective of the current study are to investigate the effects of TRD on some growth factors, photosynthetic pigments, carbohydrate contents, α -tocopherol content, glycine betaine content, proline content and PDH activity and expression level in two alfalfa cultivars under *in vitro* salt stress conditions.

MATERIALS AND METHODS

Seeds of alfalfa cultivars Yazdi and Hamedani were obtained from Pakan Bazr Company, Isfahan, Iran. Seeds were surface sterilized for 1-2 min in 95% ethanol, followed by treatment in 15% sodium hypochlorite (v/v) with 3 drops per liter of Tween-20 for 20 min the seeds were then washed 3 times with sterilized distilled water. Sterile seeds then were placed on MS medium (Murashige and Skoog, 1962) containing 0, 100, 140 mM NaCl and 1, 2, 4 mg/l exogenous Triadimefon under aseptic condition. After four weeks some growth and physiological parameters were measured.

Pigment content assay

Total chlorophyll content assay was performed according to Arnon (1949). 100 mg fresh leaves were homogenized in 1 ml 80% acetone. Homogenates were centrifuged at 4°C for 15 min (3000 rpm). Supernatants were used for the analysis of pigment. Chlorophyll content was measured according to method described by Lichtenthaler and Wellburn (1983), at 652 nm.

Water-soluble carbohydrates (WSC) and reducing sugars (RS)

All measurements were carried out on oven-dried ground leaf of plants. WSC was determined based on the phenol-sulfuric-acid method described by Dubois (1956). The reducing sugar content in the leaf was determined by Somogy method (1956).

α -Tocopherol content

α -tocopherol content was assayed as described by Backer *et al.* (1980). Five hundred milligram of fresh tissue was homogenized with 10 ml of a mixture of petroleum ether and ethanol (2:1.6 v/v) and the extract was centrifuged at 10,000 rpm for 20 min and the supernatant was used for estimation of α -tocopherol. To 1 ml of extract, 0.2 ml of 2% 2, 2-dipyridyl in ethanol was added and mixed thoroughly and kept in dark for 5 min. The resulting red color was diluted with 4 ml of distilled water and mixed well. The resulting color in the aqueous layer was measured at 520 nm. The α -tocopherol content was calculated using a standard curve.

Glycine betaine content

The amount of Glycine betaine was estimated according to the method of Grieve and Grattan (1983). The plants tissues were grounded with 20 ml of deionised water for 24 h at 25°C. The samples were then filtered and filtrates were diluted to 1:1 with 2 N H₂SO₄. Aliquots were kept in centrifuge tubes and cooled in ice water for 1 h. Cold KI-I₂ reagent was added, and the reactants were gently stirred with a vortex mixture. The tubes were stored at 4°C for 16 h and then centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was carefully aspirated with a ne glass tube. The periodide crystals were dissolved in 9 ml of 1, 2-dichloroethane. After 2 h, the absorbance was measured at 365 nm using glycine betaine as a standard and expressed in mg g⁻¹ DW.

Proline content

Free proline was determined according to Bates method (Bates *et al.*, 1973). Approximately 100 mg of dry tissue powder was homogenized in 10 ml of 3% aqueous sulfo salicylic acid for 10 min followed by ltration. Two milliliters of the ltrated samples were mixed with 2 ml of glacial acetic acid and 2 ml of ninhydrin for 1 h at 90°C. The developed colour was extracted in 4 ml toluene and measured at 520 nm against toluene. A standard curve with proline was used for the nal calculations.

Preparation of enzyme extracts

Extracts were prepared by homogenizing 0.1 g of fresh leaf in a mortar with 1.5 ml on cold potassium phosphate buffer (50 mM, pH 7.8)

containing 0.1 mM EDTA and 10% (w/w) Polyvinyl pyrrolidone (PVP). Extracts were centrifuged at 13,000 g for 20 min and the supernatant was used to determine antioxidant enzyme activities. Protein content was measured according to Bradford method (Bradford, 1976).

Enzyme assays

Proline dehydrogenase (PDH, EC 1.5.1.2) was assayed by measuring the NAD⁺ reduction at 340 nm as described by Rena and Splittstosser (1975). Two milliliter of a 100 mM sodium carbonate-bicarbonate buffer (pH 10.3) containing 20 mM L-proline, 10 mM NAD⁺ and 0.5ml enzyme extract was incubated at 25°C for 5 min, and the absorbance was measured at 340 nm. PDH was expressed as unit per mg protein (one unit is defined as a decrease in 0.001 A 340 per min).

RNA isolation

Total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. The RNA samples were quantified by absorbance at 260 nm and the purities were assessed by the 260/280 nm ratio. Four µg of each RNA sample was incubated with 1 U DNase I (Fermentas, Vilnius, Lithuania) for 30 min at 37°C. First-strand cDNA was synthesized with of four micrograms total RNA, using Revart Aid First strand cDNA synthesis KIT (Fermentas) according to the manufacturer's protocol. The reaction was achieved by incubating each sample at 70°C for 5 min, placing on ice for 3-5 sec, and incubating at 37°C for 5 min, followed by addition of reverse transcriptase and incubation at 42°C for 60 min. The reaction was stopped by heating at 70°C for 10 min. PCR amplification for PDH gene was performed in a total volume of 20 µl, containing 2 µl of cDNA, for 25 cycles in a DNA thermal cycler. After an initial denaturation step of 94°C for 2 min, each cycle included denaturation at 94°C for 2 min, annealing for 30 sec at 45°C, and extension at 72°C for 30 sec, followed by a final extension step of 72°C for 5 min. Actin gene was used as housekeeping in the same conditions. To compare the amount of different RNA samples, PCR products were examined by 1% agarose gel. RT-PCR reactions were repeated three times, using 3 different cDNA samples. PDH gene (Accession number: AY556386.1) was amplified using forward (5'-TGATCTCATGCAGACCAATA-3') and reverse (5'-AGCGTTATCATGAGCATCTT-3') primers. For amplification of actin gene forward (5'-GCTCTGCCCGTTGCTCTGATGAT -3') and reverse (5'-CCTTGGATGTGGTAGCCGTTTCT -3') primers were used. The expression level of transcripts was quantified with Image J software and analyzed in 3 replicates.

Statistical analysis of data

All experiments carried out with 3 replications and data analysis was conducted using ANOVA and Tukey test at P<0.05.

RESULTS

Data indicated that salt stress significantly (P<0.05) decreased the shoot length in both *Medicago* cultivars (Fig. 1A). TRD treatment resulted in a significant increase shoot length (P < 0.05) in 140 mM NaCl with 2 mg/l TRD while it increased root length at 100 and 140 mM salt with 1 and 2 mg/l TRD respectively (Fig. 1A, B). TRD treatment reduced the negative effect of salinity on shoot and root length and minimized the inhibitory effect of 140 mM NaCl concentration on plant growth. Our results showed that 2 mg/l TRD is the most effective level in moderating the harmful effect of salt stress and therefore, we measured the other biochemical and physiological parameters on this level of TRD.

Salt stress decreased plant total chlorophyll significantly in both cultivars, particularly in Hamedani cultivar (P<0.05) (Fig. 1C). TRD treatment resulted in unchanged amount of total chlorophyll. The treatment with TRD and salt stress enhanced total chlorophyll, significantly at 100 and 140 mM NaCl.

Carbohydrate content

The level of WSC in the leaf decreased with the elevating level of salinity in both cultivars. TRD treatment decreased WSC content while, TRD associated with salinity decreased WSC in leaf of Yazdi significantly but not Hamedani cultivar (Fig. 2A). The RS content reduced in leaf at an elevating level of salinity in both cultivars (Fig. 2B). The RS content in the leaf of Hamedani cultivar was significant lower than Yazdi cultivar (Fig. 2B). The TRD improved RS content in leaf of Yazdi cultivar but it did not affect on Hamedani cultivar significantly.

α-Tocopherol, Glycine betaine and proline content

TRD treatment increased α-tocopherol content in salt stressed plants in both cultivars. The increasing in α-tocopherol content in the leaf of Yazdi cultivar was significant and the level of α-tocopherol was higher than Hamedani cultivar (Fig. 3A). As shown in (Fig. 3B) in Hamedani cultivar salt stress supplemented with TRD didn't change glycine betaine content but it was increased in Yazdi significantly. TRD treatment increased glycine betaine content significantly in Yazdi cultivar when compared with untreated plants. In both cultivars, proline in leaf was built up by increasing salinity from 100 mM to 140 mM NaCl. Significant increase

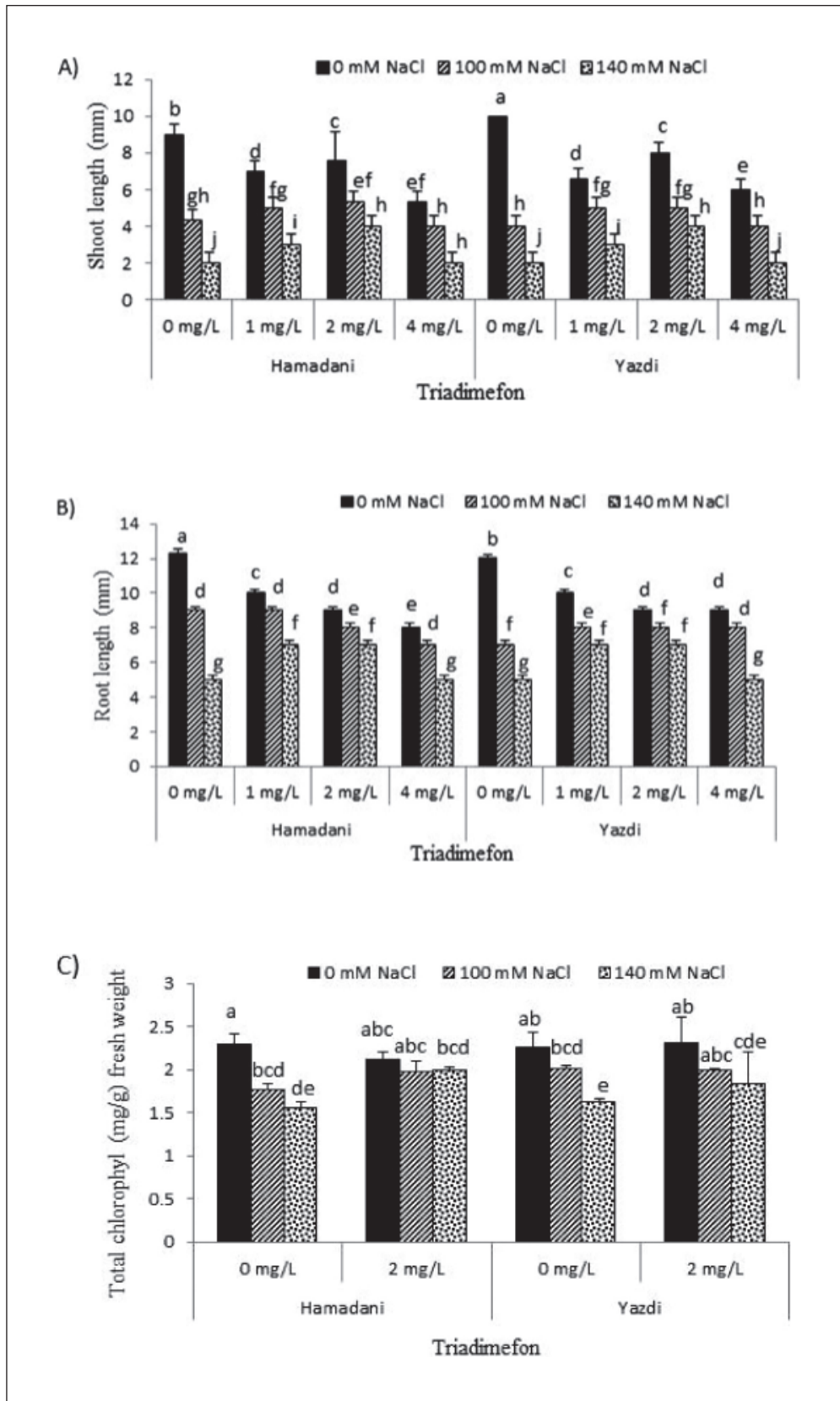


Fig. 1. Effects of salt stress and Triadimefon treatment on shoot length (A), root length (B), total chlorophyll (C), of Hamedani and Yazdi cultivars.

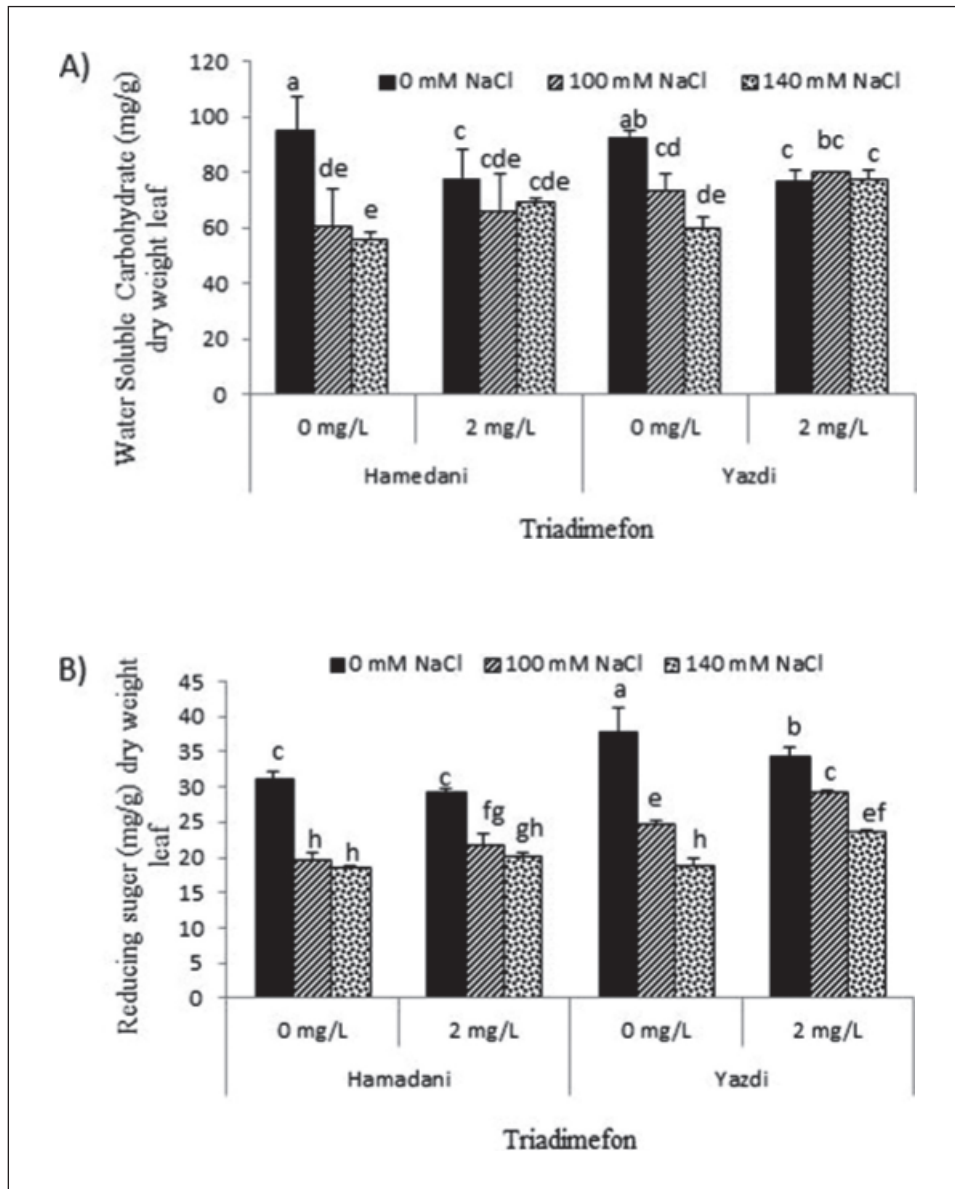


Fig. 2. Effects of salt stress and Triadimefon treatment on leaf water soluble carbohydrate (A), leaf reducing sugar (B) of Hamedani and Yazdi cultivars.

in proline content achieved in cultivar Yazdi at 140 mM NaCl while at this level of NaCl and TRD treatment proline content decreased significantly (Fig. 3C).

Activity and expression of PDH

The activity of proline dehydrogenase in the leaves of both cultivars Hamedani and Yazdi is illustrated in Figure 4A. The activity of PDH was decreased with increasing salinity as compared with the control significantly. The expression level of PDH was suppressed by NaCl (140 mM) and TRD (2 mg/l) compared to the actin gene in both cultivars significantly (Fig. 4B).

DISCUSSION

TRD treatment influenced some growth parameters in both cultivars. The effect of single TRD treatment on plant height and length was more noticeable ($P < 0.05$) compared with single salt treatment. Reduced of stem length is a consequence of Triazole-induced GA inhibition on stem elongation (Fletcher *et al.*, 2000).

Measurements of root length indicated that TRD improved the root length of Hamedani cultivar in salt treated plants. It would be associated with larger parenchyma cells and the promotion of radial cell expansion as already reported by Fletcher *et al.* (2000).

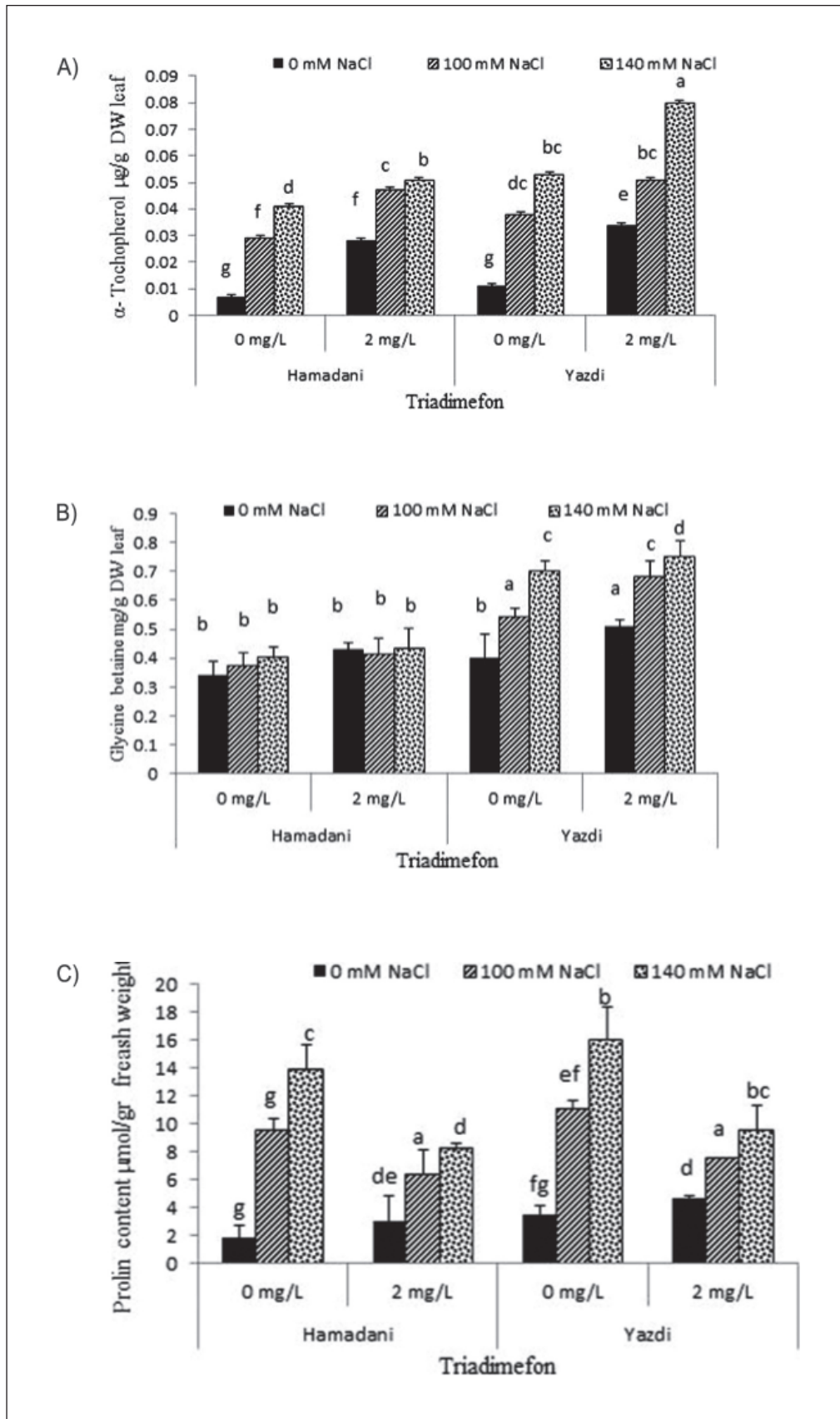


Fig. 3. Effects of salt stress and Triadimefon treatment on leaf α -tocopherol (A), glycinebetaine (B) and proline (C) content of Hamedani and Yazdi cultivars.

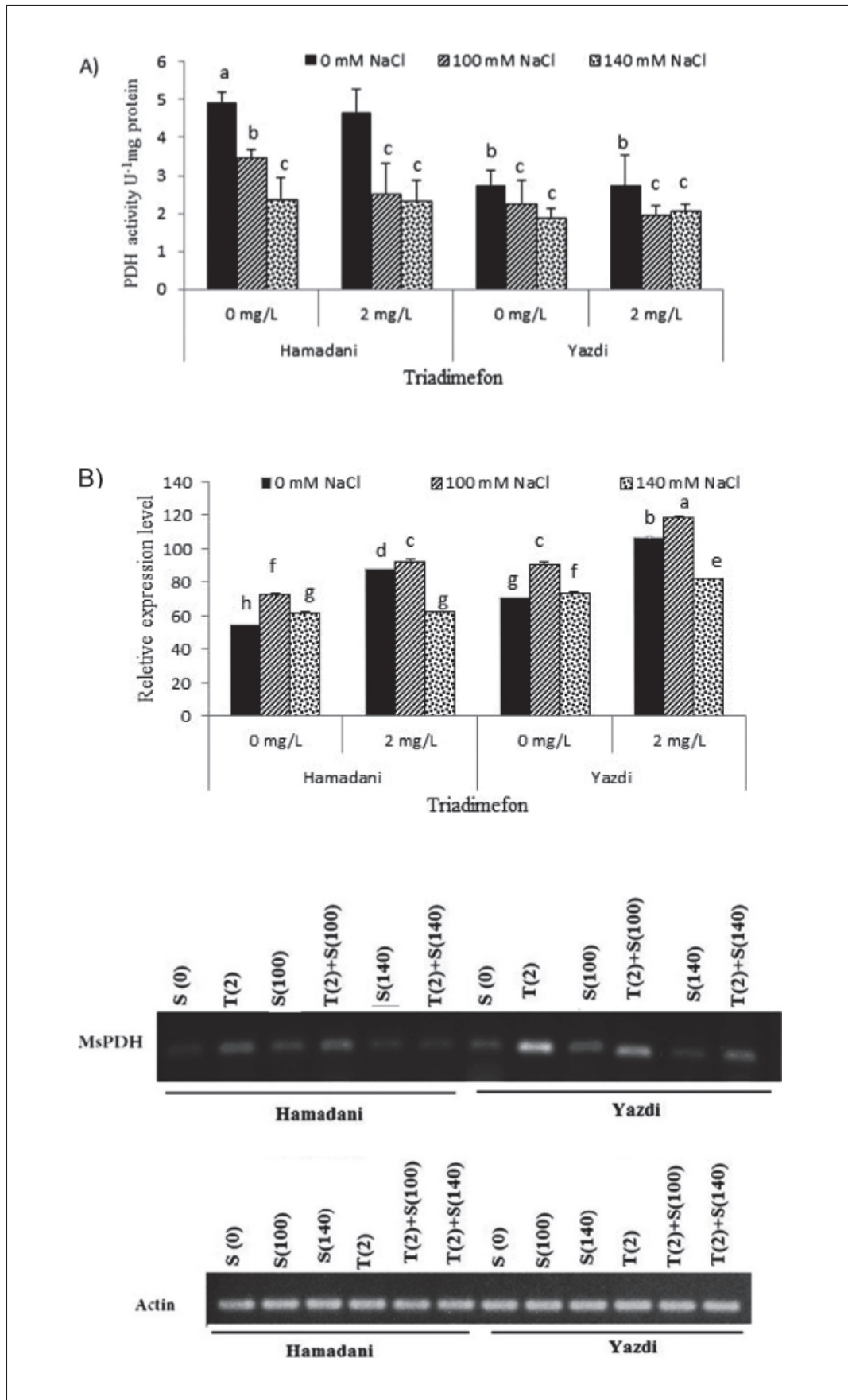


Fig. 4. Effects of salt and triadimenfon on PDH activity (A), Relative expression level of PDH gene compare to the actine gene. T: TRD, (2 mg/l), S: NaCl (0, 100,140 mM). (B), of Hamedani and Yazdi cultivars.

Photosynthesis, one of the most important metabolic pathways in plants, is a target of environmental stress. Chlorophyll is one of the major chloroplast components for photosynthesis, and chlorophyll content has a positive relationship with photosynthetic rate. The decrease in chlorophyll content under salt stress has been considered as a typical symptom of oxidative stress and may be due to the result of pigment photo-oxidation and chlorophyll degradation by chlorophyllase activity. Photosynthetic pigments are important to plants mainly for harvesting light and production of carbohydrate. In the present study, the total chlorophyll in Hamedani cultivar was significantly increased by TRD treatment. It has been reported that several environmental stresses can cause an excess amount of toxic reactive oxygen species (ROS) (Scandalios, 1993). Since TRD enhances the free radical scavenging capacity of salt treated plants (Senaratna *et al.*, 1988), consequently, chlorophyll content might be kept away from ROS or TRD may decrease the activity of chlorophyllase enzyme. Comparing of two *Medicago sativa* cultivars in chlorophyll content after TRD treatment might be a genotype dependent response.

The analysis of data showed that TRD treatment increased the amount of WSC and RS in NaCl treated plant. In the course of salt and drought stress, accumulation of compatible soluble such as proline, polyamines and carbohydrate is claimed to be an effective stress tolerance mechanism (Martin *et al.*, 1993). It can be speculating that carbohydrate accumulation in TRD-treated plants was correlated with the increasing salt tolerance.

α -Tocopherol content increased in both cultivars under salt stress when compared with control plants. TRD treatment further enhanced α -tocopherol content in both *Medicago* cultivars. α -Tocopherol (Vitamin E) is lipophilic antioxidant synthesized by plants and are major lipid soluble antioxidant present in the polyunsaturated fatty acid (PUFA) enriched membranes of chloroplasts and it has been proposed to be an essential component of plastid antioxidant network. α -Tocopherol interacts with the polyunsaturated acyl groups of lipids, stabilize membranes and scavenge ROS (Wang and Quinn, 2000). It has been reported that triazole treatments such as PBZ (paclobutrazol) increase α -tocopherol content in wheat under stress condition (Kraus *et al.*, 1995). Fletcher *et al.* (2000) has also reported that the stress protective effect of triazole against stress is mediated by an increase in antioxidant (Vitamins C and E) contents. These reports supported our data where level of α -tocopherol was increased due to salt stress in both *Medicago* cultivars.

Glycine betaine is known to accumulate in response to stress in many plants, e.g. sorghum (Yang *et al.*, 2003), and in peanut (Girija *et al.*, 2002). Accumulation of glycinebetaine has also been found in reducing the adverse effects of drought stress in sunower (Yang and Lu, 2005). Glycine betaine prevented disintegration of grana stacking and intergranal lamellae and destruction of mitochondria due to NaCl stress. Glycine betaine induces production of additional vacuoles in root cells, which resulted in a greater accumulation of Na^+ in the root and a decrease in its transportation to the shoot (Yang *et al.*, 2003). Similar result was obtained from our data. We demonstrated that NaCl treatment led to a significant increase in the level of glycine betaine in Yazdi cultivar.

Proline is a candidate molecule that contributes to the increase in biomass, osmotic regulation and stress response in plants. Under NaCl treatment proline accumulation was observed in both alfalfa cultivars. It should be kept in mind that proline accumulation under salt stress occurs either in salt sensitive or salt tolerant plant. However, the highest level of proline in Yazdi cultivar at 140 mM NaCl might be a suitable indicator for recognizing a salt tolerant cultivar. Co-treatment of plant with TRD and NaCl changed proline content. Based on the results, it can be speculating that TRD changes the response of plant to salt stress. This is not only by increasing of proline but also with other processes such as increasing antioxidant enzymes activity, stabilizing of sub-cellular structures such as membranes and proteins and free radicals scavenging under stress. A similar mechanism for proline function in response to salt stress has been reported by Manivannan *et al.* (2008).

Proline degradation to glutamate occurs in the matrix side of the inner mitochondrial membrane through the sequential action of proline dehydrogenase (PDH) and P5C-dehydrogenase (P5CDH). We observed that *Medicago sativa* cultivar Yazdi has a tendency to maintain or mainly to decrease PDH activity after salt treatment. Decreasing PDH activity was more notable at 140 mM NaCl compare to the untreated plants. Hamedani showed slightly lower PDH activity after salt stress compared to Yazdi. It might be due to difference in genotype. Combination of TRD and NaCl showed no significant difference in PDH activity. Similar observation has been supported for PDH activity in response to salinity by Wang *et al.* (2011). Rahnama and Ebrahimzadeh (2004) reported that salt stress caused a reduction in the activity of PDH in all cultivars of *Solanum tuberosum*. Such reduction in PDH activity and the simultaneous increase in proline level have also been reported in *Brassica juncea* under salt stress (Madan *et al.*,

1995). In the present study, expression of PDH was increased under salt stress in both cultivars. However at 140 mM NaCl the expression level of this enzyme was decreased. It can be a consequence of high salt concentration of the medium on plant growth and development. Our data showed that alfalfa subjected to NaCl and TRD increased expression of PDH gene. Treatment of alfalfa with TRD slightly increased PDH transcript level. Our results indicated that increasing level of proline might be related to increasing level of PDH transcripts.

It can be concluded that TRD 2 mg/l was found to enhance plant growth and development by increasing of total chlorophyll, carbohydrate, α -tocopherol, glycine betaine content and induced PDH activity and PDH transcript level under *in vitro* salt stress condition.

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