PROGRESS IN BIOLOGICAL SCIENCES

Improvement of some physiological responses of alfalfa *(Medicago sativa* L.) under *in vitro* salt stress using Triadimefon

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Abstract

This study was carried out to investigation the possibility of using Triadimefon (TRD) in order to decrease the adverse effects of salt stress on *Medicago* plant. Triadimefon is a member of Triazol compounds which enhances stress tolerance through physiological processes. Two cultivars of *Medicago sitiva* including Hamedani and Yazdi were used in this study. Plants were treated with 1, 2 and 4 mg/l TRD and 0, 100 and 140 mM NaCl. Salinity reduced fresh and dry weight of plants in both cultivars with the result of higher reduction in cv. Hamedani significantly. Salt stress also increased proline, MDA and H_2O_2 contents and also the activity of SOD and P5CS, and the level of P5CS transcripts, while decreased the activity of CAT and POX in both cultivars. When plants were treated with TRD and NaCl, less proline, MDA and H_2O_2 accumulation occurred. Interaction of NaCl and TRD increased SOD but reduced P5CS and CAT activity. Both NaCl and TRD treatments increased P5CS transcription in cultivar Yazdi while showed less effect in Hamadani cultivar. Interaction of TRD and NaCl alleviated the negative effects of salt stress on plant growth, especially at 2 mg/l TRD. The results of the present study showed that 2 mg/l TRD was the most effective level to alleviate the negative effects of NaCl treatment on *Medicago sativa* plants.

Keywords: Antioxidant enzyme, Medicago, Salt stress, Triadimefon

Introduction

Soil salinity is one of the most hazardous environmental stresses, which can induce an oxidative stress to plants (1). Salt stress alters various biochemical and physiological responses in plants, and affects almost all plant processes including photosynthesis, growth and development (2). The major effect of salinity is the inhibition of plant growth possibly by reducing enzymatic activities and biochemical constituents (3). High salinity is known to cause hyper osmotic effects in plants leading to membrane disorganization and metabolic toxicity (4).

Salt inhibits plant growth by inducing oxidative stress through an increase in reactive oxygen species (ROS), such as superoxide ($O^{\cdot}{}_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\cdot}$), which disturb in turn the balance between the production of ROS and the quenching effects of antioxidant enzymes thus, imparting oxidative damage to lipids, proteins, and nucleic acids (5). To overcome the negative consequences of salt stress, plants have evolved various protective mechanisms either to reduce or to completely eliminate ROS. One of the protective mechanisms is changes in enzymatic anti oxidant activity, which operate with a sequential and simultaneous action of many enzymes such as superoxide dismutase, peroxidase, and catalase (6) which all react with ROS to keep it at low levels. The salt tolerant plants have an efficient antioxidant system for effective removal of the ROS (7).

Proline accumulation is a common metabolic response when higher plants are exposed to water deficits and high salt. Proline acts as an osmolyte that not only stabilizes protein structures, but also acts as the regulator of cellular redox potential (8).

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The ability of proline accumulation under stress is often associated with stress tolerance in various plant species. Proline is synthesized by either glutamate or arginine/ornithine pathways in higher plants. The glutamate pathway is the major route for proline synthesis during stress. Under the glutamate pathway, proline is synthesized from glutamic acid via the intermediate glutamic semialdehyde (GSA) and 1-pyrroline-5-carboxylate (P5C). 1-pyrroline-5-carboxylate synthetase (P5CS), which catalyzes the first two reactions of proline biosynthesis (9).

Application of growth regulators has been reported to reduce the adverse effects of salinity (10, 11). The use of plant growth regulators has been reported to result in a significant increase in the growth and yield of many crops under stress condition (12). Triazole compounds such as triadimefon (TRD), [1-(4-chlorophenoxy)-3,3-dimethyl-1-(1H-1,2,4-triazole-1-yl)-2-butanone), paclob-utrazol (PBZ), uniconazole (UCZ), are widely used as fungicides and they also possess varying degrees of plant growth regulating properties and have been called 'plant multi-protectants' because of their ability to enhance tolerance in plants against environmental and chemical stresses (10, 12, 13). The most pronounced effect of triazole on plants is a reduction in height (14). There are also evidences indicating that triazoles may increase cold and heat resistance (12, 15), and drought tolerance (16). Protection of plants from apparent unrelated stress by triazole is mediated by a reduction in free radical damage and an increase in the antioxidant potential (17). The plant growth regulating properties of TRD are mediated by interference with the isoprenoid pathway and subsequent shift in the balance of important plant hormones, including gibberellins, ABA and cytokinins (14). However, the exact mechanism of TRD on plants under salt stress has not been studied much. Some researchers reported that TRD reduced the effect of salt stress on guava and grapes (18, 19). It was hypothesized that TRD-treated plants had a better quality of growth under salt stress than non-treated plants (20).

Medicago sativa is a leguminous plant species, which is relatively salt tolerant and has been grown for a variety of purposes such as soil improvement, animal feed, medicinal uses and suitable foliage (21). There are no reports on the effects of exogenous TRD enhancing alfalfa tolerance to salt stress. Based on a few reports of TRD application for plants as a new compound with plant growth regulator properties, the mechanism of this compound and its effect on stress tolerance in plants is rather sketchy. Therefore, our goal was the effects of this compound on salt tolerance of *Medicago* plants. In the present study, an attempt was made to evaluate the salt stress ameliorating ability of TRD in two cultivars of *Medicago sativa* (Yazdi and Hamedani) under *in vitro* salt stress.

Materials and methods

Seeds of two alfalfa cultivars, Yazdi and Hamedani, were obtained from Pakan Bazr Company of Isfahan, Iran. Seeds were surface sterilized for 1-2 min in 95% ethanol, followed by treatment in 15% sodium hypochlorite (v/v) with 0.1% Tween-20 for 20 min. Then the seeds were washed 3 times with sterilized distilled water. Sterile seeds were placed on MS (22) medium containing 0, 100, 140 mM NaCl and/or 1, 2, 4 mg/l TRD under aseptic condition. After four weeks, plants were harvested to measure different parameters with 3 replications per each treatment.

Analysis of growth parameters

Fresh weight of plants was measured after 4 weeks. Then the samples were dried in oven at 70°C for 48 h. Then the dry weights (DW) were determined.

Proline

Free proline was determined according to Bates et al. (1973) (23).

Lipid peroxidation

Lipid peroxidation was determined by measuring malondialdehyde (MDA) formation according to Heath and Packer (1968) (24). Fresh leaves (300 mg) were homogenized with 2.5 ml of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged for 10 min at 10,000 rpm. For each 1 ml of the aliquot, 4 ml of 20% TCA containing 0.5% thiobarbituric acid (TBA) was added. The mixture was heated at 95ºC for 30 min then cooled quickly on an ice bath. Afterwards, the mixture was centrifuged for 15 min at 10,000 rpm and the absorbance of the supernatant was measured at 532

nm. Measurements were corrected for non specific turbidity by subtracting the absorbance at 600 nm. The concentration of MDA was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

H_2O_2 content

Hydrogen peroxide content was determined according to Velikova et al. (2000) (25). Leaf fresh tissue (0.1 g) was extracted with 3 ml TCA (0.1% w/v) in an ice bath and centrifuged at 12000 g for 15 min. Then, 1 ml of the supernatant, 1 ml phosphate buffer (pH 7.0) and 2 ml of 1 M KI, were added respectively and the absorbance of the mixture was measured at 390 nm. The H_2O_2 content was calculated using the extinction coefficient of 0.28 mM^{-1} cm^{-1}

Preparation of enzyme extracts

The extracts were prepared by homogenizing 0.5 g of fresh leaf in a mortar with 1.5 mL of cold potassium phosphate buffer (50 mM, pH 7.8) containing 0.1 mM EDTA and 10% (w/w) Polyvinyl pyrrolidon. The extracts were centrifuged at 13,000 g for 20 min and the supernatant was used for enzyme assay. Total protein content was also measured according to Bradford (1976) (26).

Enzyme assay

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured based on the method of Beauchamp and Fridovich (1971) (27). About 1 ml of the reaction mixture containing 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 75 µM nitro blue tetrazolium (NBT), 2 µM riboflavin, and 50 µl of the enzyme extract was poured to the test tube. After adding Riboflavin, the reaction was initiated by exposing the tubes under fluorescent white light (approx. 500 Lux) for 10 min. Product reaction was measured at 560 nm. The volume of the supernatant resulting in 50% inhibition of the reaction was taken as one enzyme activity unit.

Peroxidase (POX, EC 1.11.1.7) activity was determined by monitoring the formation of tetraguaiacol from guaiacol at 470 nm (ε = 26.6 $mM^{-1}cm^{-1}$. The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0), 20.1 mM guaiacol, 12.3 mM H_2O_2, and 50 µl of the enzyme extract in a final volume of 1 mL (28).

Catalase (CAT, EC 1.11.1.6) activity was assayed by following the reduction of H_2O_2 (ε = 39.4 $mM^{-1}cm^{-1}$) at 240 nm according to the method of Aebi (1984) (29). One unit of CAT was defined as 1 µmol H_2O_2 min^{-1} mg^{-1} protein.

P5CS (EC 2.7.2.11/1.2.1.41) activity was determined by monitoring the consumption of NADPH. Two milliliters of the reaction mixture containing 75 mM Glu, 100 mM Tris-HCl (pH 7.2), 20 mM $MgCl_2$, 5 mM ATP, 0.4 mM NADPH and 0.5 ml enzyme extract was incubated at 37°C for 20 min and then, the absorbance was recorded at 340 nm (30).

RNA isolation

Total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. First-strand cDNA was synthesized with four micrograms total RNA, using Revart Aid First strand cDNA synthesis KIT (Fermentas) according to the manufacturer's protocol. PCR amplification for the P5CS 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 s at 60°C, and extension at 72°C for 30 s, followed by a final extension step of 72°C for 5 min. Actin was used as housekeeping gene. 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. The P5CS gene (GenBank: CAA67069.1) was amplified using forward (5′-CATCCCTGTTTCTCTCCACC-3′) and reverse (5′-CCATCTCGCGTACATCAAC C-3′) primers. To amplify the actin gene forward (5′-GCTCTGCCCGTTGCTCTGATGAT-3′) and reverse (5′-CCTTGGATGTGGTAGCCGTTTC T-3′) primers were used. The expression level of transcripts was quantified with Image J software (rsb.info.nih.gov/ij/docs/install/) and then analyzed in 3 replicates.

Statistical analysis of data

All experiments were carried out in three replicates and analyzed using ANOVA based on Tukey test

at P<0.05. In all figures similar letters are not significant based on Tukey test.

Results

In both cultivars, the fresh weight (FW) and the dry weight (DW) of shoot and roots were reduced at 100 and 140 mM NaCl (Fig. 1A, B, C, D). The negative effect of salinity on the fresh and the dry weight of plants in Hamedani cultivar was more than in Yazdi, particularly at 140 mM NaCl. TRD treatment reduced the negative effect of salinity on FW and DW of shoot and roots. Regarding FW and DW, the role of TRD in controlling salinity effect on Hamedani was more considerable than Yazdi cultivar. Individual treatment of NaCl significantly decreased shoot FW in both cultivars (P<0.05), while TRD improved plant growth by increasing salinity level (Fig. 1A, B, C, D).

Since our results showed that 2 mg/l TRD was the most effective level in alleviating the adverse effect of salt stress (Fig. 1A, B, C, D), the other parameters were measured at this concentration.

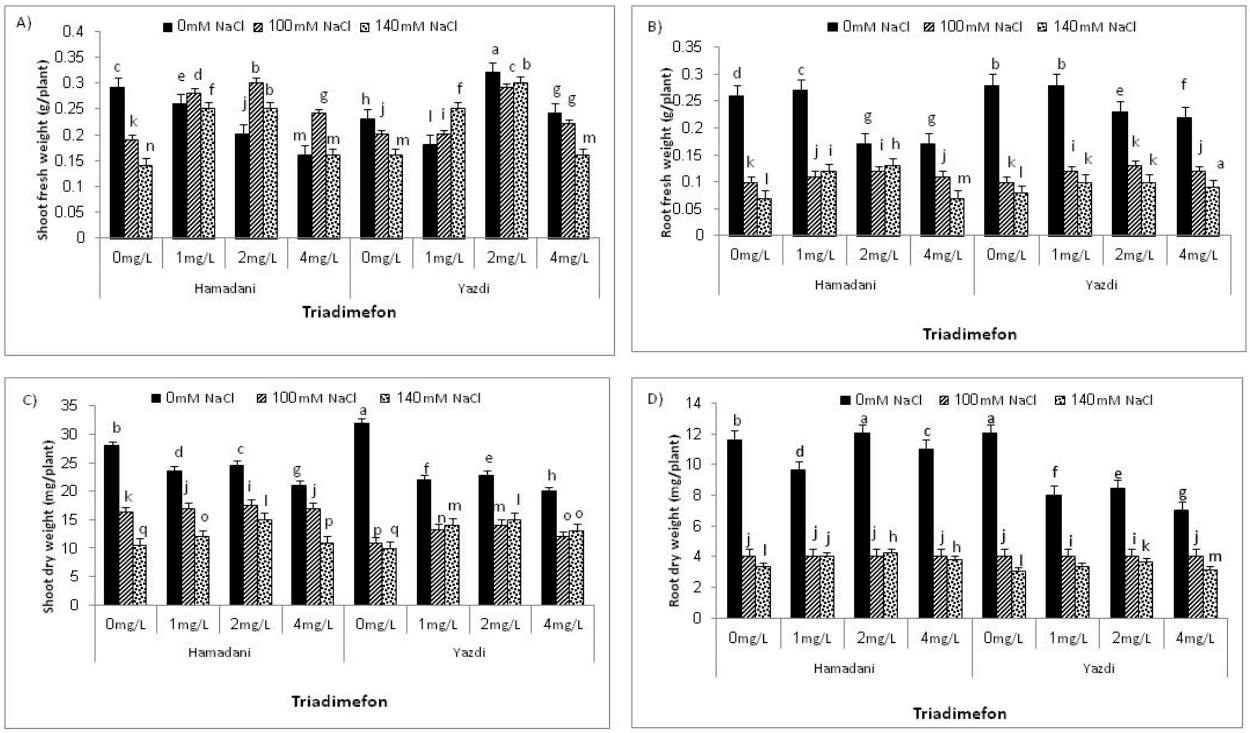

Figure 1. Effect of NaCl and Triadimefon treatments on shoot fresh **(A)**, root fresh **(B)**, shoot dry **(C)** and root dry weighs **(D)** in *Medicago sativa* cultivars.

Proline content

In both cultivars, proline accumulation was enhanced by increasing salinity from 0 to 140 mM NaCl. A significant increase in proline content was observed in cultivar Yazdi at 140 mM NaCl. In the non-salinized plants, TRD treatment significantly increased proline content of both cultivars. The interaction of TRD and NaCl treatment significantly reduced proline accumulation compared to the same NaCl level without TRD (Fig. 2).

Lipid peroxidation

Lipid peroxidation is illustrated as MDA content in Fig. 3A. MDA content was significantly increased in 140 mM of NaCl in Hamadani cultivar while no significant increase was observed in other treatments compared to the control plant. TRD had no considerable effects on MDA contents and no significant changes were observed between different concentrations of NaCl with TRD treatment.

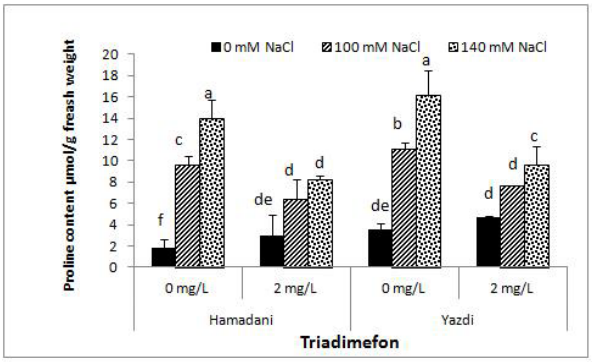

Figure 2. Effect of NaCl and Triadimefon on proline content of *Medicago sativa* cultivars.

H_2O_2 content

The H_2O_2 content was measured to determine some ROS status induced in alfalfa plants upon exposure to salinity and TRD treatments. Salt stress as a single treatment significantly increased H_2O_2 content in both cultivars, with the highest increase in Yazdi cultivar at 140 mM NaCl (Fig. 3B). Hamedani cultivar accumulated lower H_2O_2 than Yazdi cultivar. TRD decreased the H_2O_2 level less than the control plants of Yazdi cultivar. The interaction of TRD and the salinity reduced H_2O_2 accumulation compared to the same NaCl level without TRD treatment (Fig. 3B).

Antioxidant enzymes

As the results showed, in both cultivars, CAT activity was reduced by increasing salinity from 100 mM to 140 mM NaCl. (Fig. 4A). TRD treatment significantly decreased CAT activity either with or without NaCl. POX activity in stressed plants showed almost similar pattern to CAT enzyme (Fig. 4B). Single TRD treatment had no significant effects on POX activity. The interaction of TRD and salt stress significantly decreased POX activity. The POX activity in Hamadani cultivar was less than Yazdi cultivar (Fig. 4B).

SOD activity was increased due to the increasing salt concentration in both cultivars, with more increase in Yazdi cultivar (Fig. 4C). In non-salinized plants, TRD treatment enhanced SOD activity. The interaction of TRD and 140 mM NaCl regulated the negative effects of salinity (Fig. 4C). The effects of salt stress and TRD on P5CS activity in two alfalfa cultivars were studied (Fig. 4D). Salt stress increased P5CS activity in both cultivars with the highest activity at 140 mM NaCl. In Yazdi cultivar, P5CS activity was higher than Hamadani cultivar. Under NaCl with TRD treatments, P5CS activity decreased in both cultivars compared to the same salinity level.

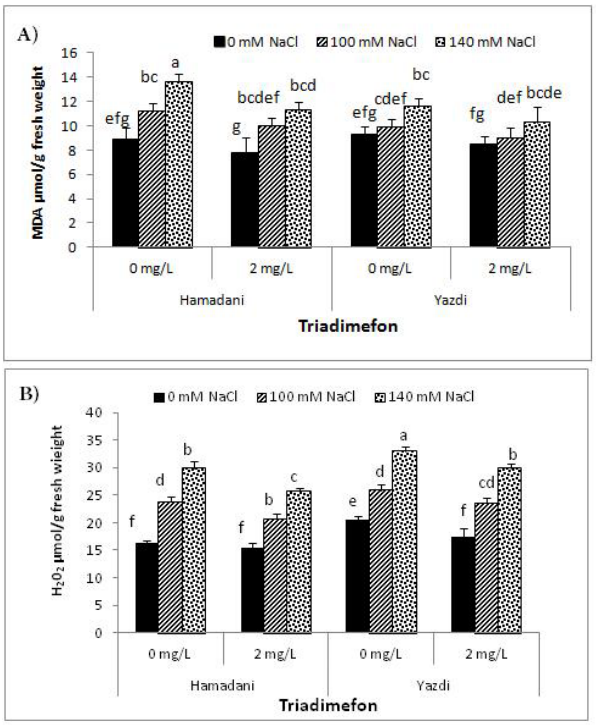

Figure 3. Effect of NaCl and Triadimefon on leaf MDA **(A)**, H_2O_2 contents **(B)** of *Medicago sativa* cultivars.

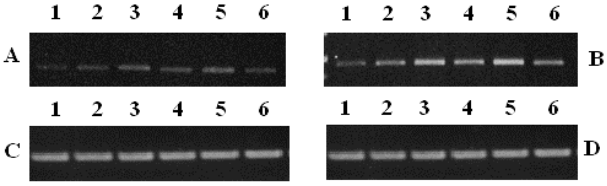

Figure 4. Effect of NaCl and Triadimefon on leaf catalase **(A)** peroxidas **(B)** superoxide dismutase **(C)** pyrroline-5-carboxylate synthetase **(D)** of *Medicago sativa* cultivars.

Expression of the *P5CS* gene

The expression level of P5CS was significantly induced by NaCl treatment ($P<0.05$), as compared to the control in both cultivars (Fig. 5A, B). NaCl treatment with TRD decreased the expression of P5CS in Yazdi cultivar.

Discustion

In this study, the physiological and growth responses of the two cultivars of alfalfa to salt stress and TRD were examined. Results demonstrated that TRD treatment and salt stress affected some plant growth parameters. As a general responses of plant to salt stress, plant growth was affected negatively in various ways such as osmotic shock, specific ion and nutritional imbalance, probably all occurring simultaneously (31). Also, initial growth inhibition in saline environment was induced by the decreased water potential of root medium due to the high salt concentration (32). In our study, data of growth parameters showed that, Hamedani cultivar was slightly more sensitive to salt stress than Yazdi, especially at 140 mM NaCl. Increasing of NaCl concentration slowed water uptake by plants, thereby a decrease in water content was followed by reduction of fresh weight in both cultivars, particularly in Hamadani. In agreement with our result, growth reduction and decreasing of water content induced by water stress have been universally observed even in the tolerant plants (33, 34). TRD application reduced the negative effect of salinity on FW of shoot in both cultivars. In Hamedani cultivar, one positive effect of TRD treatment was increasing FW of the roots by increasing salinity against non-treated plants by TRD (Fig. B). We found that, decreasing of dry weight (DW) in shoot was correlated with decreasing of plant height in TRD treated plants. It is hypothesized that the induced-resistance due to salinity in TRD treated plants was probably

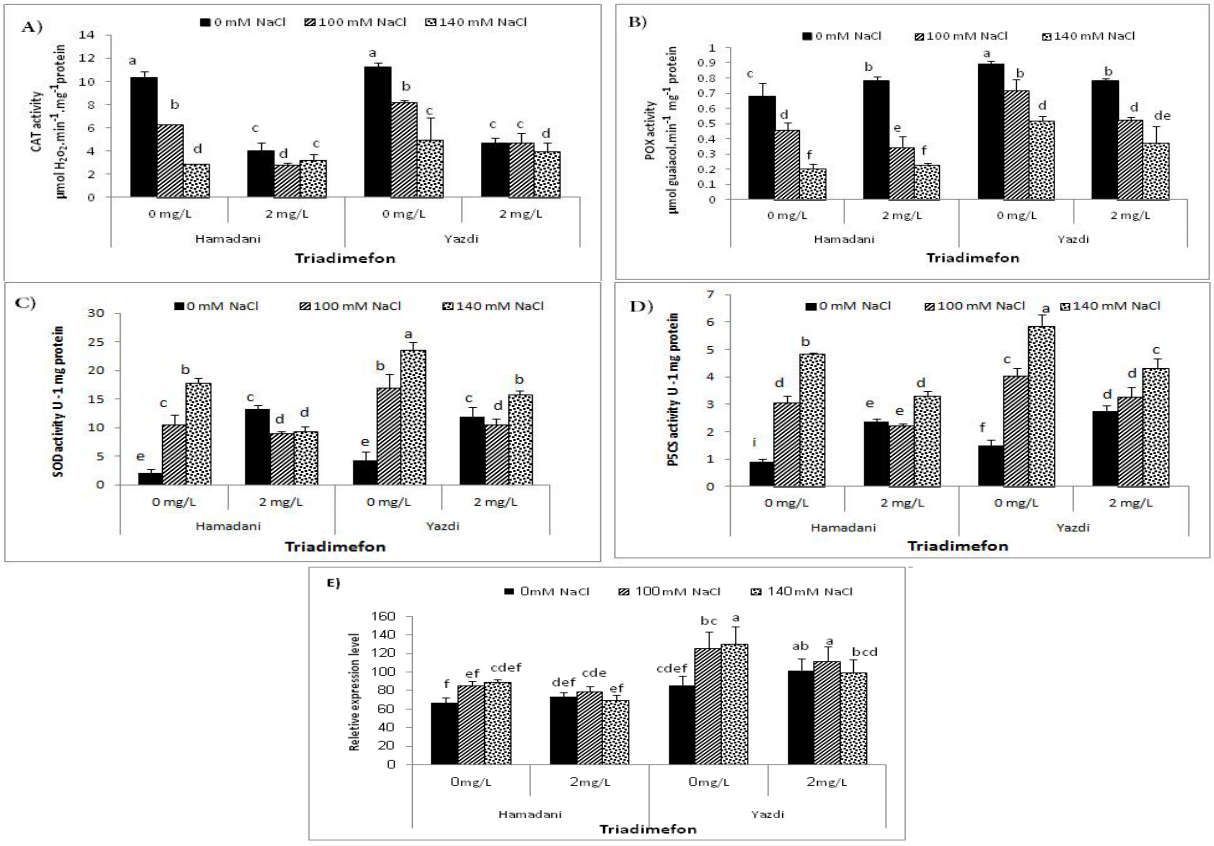

Figure 5. Effect of NaCl and Triadimefon on relative expression of P5CS gene in Hamedani **(A)** and Yazdi **(B)** cultivars of *Medicago sativa*, Actin gene as internal control **(C)** and **(D)** (1): control (without treatment); (2): 2 mg/l Tridaimefon; (3): 100 mM NaCl; (4): 100 mM NaCl, 2 mg/l tridaimefon; (5): 140 mM NaCl; (6): 140 mM NaCl, 2 mg/l Tridaimefon. Expression was quantified with Image J software **(E)**. Product volumes are means ± SE from 3 independent experiments.

Sulfate salts

The effect of metals as sulfate salts on the clavulanic acid production was shown in Figure 2. Only $MnSO_4$ increased clavulanic acid production. The difference of antibiotic concentration in the media containing 0.41 mM Mg^{2+} and control was not significant ($P<0.05$). In the media containing Ca^{2+}, Co^{2+}, Cu^{2+} and Zn^{2+}, less clavulanic acid was produced than that of control. Addition of $MgSO_4$, $CuSO_4$ and $FeSO_4$ increased the biomass concentration, while other metal sulfate salts decreased it, significantly. In the media containing sulfate and chloride salts, pH of the fermentation media was more and less than that of control, respectively.

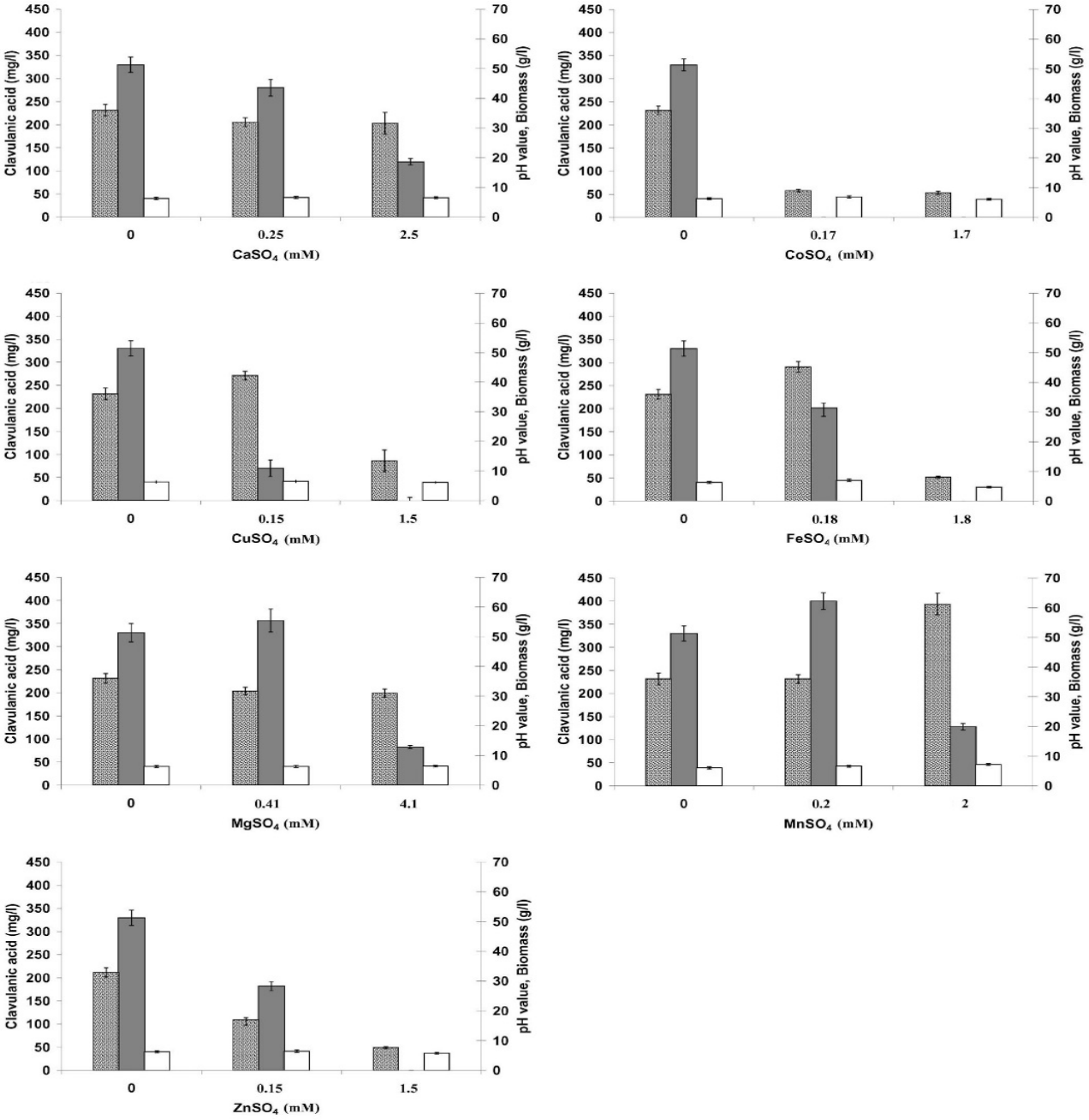

Figure 2. The effect of various metals as sulfate salts on the clavulanic acid production by *Streptomyces clavuligerus* DSM 738 in the basal medium containing various concentrations of the metal salts. A control fermentation medium in the absence of any metal supplement was also included (▩biomass (g/l), ■clavulanic acid, □ pH).

suggested that triazoles induce stress tolerance in plants at least in part by increasing of antioxidant activity.

We observed that *Medicago sativa* cultivar Yazdi had a tendency to maintain or mainly to increase P5CS activity after salt treatment. Combination of TRD and NaCl increased both the P5CS activity and the activity pattern of this enzyme was similar to salt treated plant without TRD. Increasing of P5CS activity coincides with increasing of proline content. Similar results have been reported for P5CS activity in response to salinity (54). Proline biosynthesis is a typical biochemical adaptation in plants when subjected to stress condition (55). In maize seedlings, the treatments induced proline accumulation by activation of the biosynthetic pathway, including P5CS and OAT (ornithin aminotransferase) (56). In *Medicago truncatula* (57) and in young *Arabidopsis* leaves (58), induction of OAT (ornithin aminotransferase) mRNA by osmotic stress has been reported, suggesting that both glutamate and the ornithine pathways may contribute to proline accumulation under stress condition. Wang (2011) proved that in the proline biosynthesis, the glutamate pathway plays a leading role under salt stress rather than the ornithine pathway.

It was postulated that glutamate pathway of proline synthesis is predominant under osmotic stress in plants (59). When proline synthesis is induced by osmotic stress, the P5CS gene plays a role as a rate-limiting enzyme in the proline biosynthetic pathway in plants. A correlation between transcript level of the P5CS gene and proline content was shown in a number of plants (60).

In the present study, expression of P5CS was increased under salt stress in both cultivars. However at 140 mM NaCl the expression level decreased, and this can be interpreted as harsh effect of high salt concentration on plant growth and development.

Our data (Fig. 5) showed that alfalfa subjected to NaCl with TRD declined expression of P5CS gene while treatment of alfalfa with TRD alone slightly increased P5CS transcript level, especially in Yazdi cultivar. These results indicated that alternations in the levels of P5CS transcripts are responsible for the altered proline content in the salt treated plants. Armengaud (2004) reported that in response to osmotic stress, the glutamate biosynthesis pathway is strongly activated through MtP5CS2 transcript accumulation in seedlings. Elevated levels of proline caused by over expression of P5CS confirms the enhanced tolerance to salt stress in plants (61).

From these results, it is proved that, interaction of salinity with TRD had an effective role in decreasing MDA and H_2O_2, proline contents, SOD and P5CS, CAT and POX activity, the level of P5CS transcripts particularly at 140 mM NaCl. The decreased MDA and H_2O_2 may imply an improved salt tolerance in alfalfa plants using TRD.

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