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Comparative responses of two *Trigonella* species to salinity and drought stresses *in vitro*

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ABSTRACT

Effects of salinity and drought on growth, contents of proline, malondialdehyde (MDA), protein and activity of antioxidative enzymes were studied in two *Trigonella* species. Seeds and explants of *T. foenum-graecum* and *T. aphanoneura* were grown on Murashige and Skoog medium (MS) complemented with iso-osmotic concentrations of NaCl (0, 100, 150 mM) and mannitol (0, 180, 275 mM). Growth and relative water content (RWC) of seedlings and calli decreased by increasing of salinity and drought in both species. In contrast to that of calli, proline and protein contents increased in seedlings of both species under both stresses. The increase of proline content in seedlings of *T. aphanoneura* was higher than that of *T. foenum-graecum* under NaCl stress. MDA content in seedlings of *T. aphanoneura* was higher than that of *T. foenum-graecum* and increased in both species under salinity. Among antioxidative enzymes, catalase (CAT) activity increased continuously in seedlings of *T. aphanoneura* comparing to that of *T. foenum-graecum.* Similar increasing trends were obtained regarding CAT activities in calli of both species under both stresses. Increase in activities of SOD, CAT and POX was observed in calli of both species under stress. It seems that undifferentiated calli respond more regularly to both stresses. Finally, higher proline content and lower amount of MDA could be considered as criteria for higher tolerance of *T. foenum-graecum* seedlings against osmotic stresses.

Keywords: antioxidant enzymes, callus, lipid peroxidation, tissue culture, tolerance.

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Introduction

Crops grown in arid and semi-arid regions are exposed to environmental factors such as drought and salinity. Soil salinity and drought are the most important constraints that affect plant growth worldwide and can reduce plant production (1, 2). Understanding the biochemical and molecular responses of plants to abiotic stresses is essential for deciphering plant resistance mechanisms to osmotic stress. When plants are subjected to various abiotic stresses such as drought and salinity, some reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydrogen radicals (OH) are produced. ROS is responsible for various stress-induced damages to macromolecules and cellular structure (3, 4). Strategy of plants to overcome oxidative damage is to produce non-enzymatic antioxidants (ascorbate, gluthatione, tocopherols, carotenoids, phenols) and antioxidative enzymes including peroxidase (POX), catalase (CAT), superoxide dismutase (SOD), glutathione reductase and polyphenol oxidase (PPO) (5, 6). Also, Jagesh et al. (7) reported that osmoprotectants or compatible solutes such as proline act in osmotic adjustment; they can stabilize proteins and membranes under various stresses. *In vitro* tissue culture techniques provide a controlled and uniform environment for studying physiological and biological processes in plants, particularly at the cellular level under salt-induced osmotic stresses (8, 9, 10) and drought- induced osmotic stresses (11).

Trigonella L. is a large genus with close to 135 species belonging to family Fabaceae. Two important species of this genus are *Trigonella foenum-graecum* (Fenugreek) and *Trigonella aphanoneura.* Fenugreek, a flowering annual plant is cultivated in the most regions of the world such as Iran, India, China, Ukraine, Turkey, etc. for its medicinal values (12). In many countries this species is grown in arid and semi-arid regions, where soil salinity is an important problem. The most species of *Trigonella* are distributed in the dry regions in E. Mediterranean, W. Asia, S. Europe, N. and S. Africa, with only one species being present in S. Australia (13). Rechinger (14) reported the presence of 58 species of *Trigonella* in Iran. Fenugreek leaves and seeds are consumed as medicine in different countries around the world. These species play an effective role in curing or in controlling many diseases such as, diabetes, blood sugar and cholesterol level and cancer. Also, the *Trigonella* species are important fodder crops. Salinity, drought, chilling and heat are serious threats for the *Trigonella* species in many regions of Iran (15).

This study was carried out to test the hypothesis that salinity and drought induces different changes in osmotic relations and antioxidative responses in two species of *Trigonella in vitro*. Thus, this study may contribute for a better understanding of the responses of calli and seedlings of *Trigonella* to increasing salinity and drought and improve knowledge of the stress physiology in these important plants.

Materials and Methods

Plant materials and stress treatments

Seeds of *T. foenum-graecum* L. and *T. aphanoneura* Rech. f. were obtained from Alborz Institute in Karaj, Iran. Seeds of both species were manually dehusked and the surface was sterilized in 15% (v/v) sodium hypochlorite solution for 15 min, followed by 3 washes with sterile distilled water. Then, twenty sterilized seeds were placed on petriplates containing MS media (16) and different treatments of NaCl and mannitol, separately. NaCl and mannitol (water-deficit

stress) in the culture media were adjusted to 100 and 150 mM, and 180 and 275 mM, respectively. These various iso-osmotic concentrations corresponded to osmotic potentials of -0.7, and -0.9 MPa determined by a vapor pressure osmometer (Wescore, 5520, United States). The control medium has an osmotic potential of -0.4 MPa. The experiment was arranged with four replicates and seedlings were maintained in a growth chamber for 30d. For callus initiation, two weeks old seedlings were used as source of explants. Leaf explants (0.3 cm^2) grown on MS medium were transferred to the same solid MS medium supplemented with 1 mg l^{-1} 2, 4-D and 0.5 mg l^{-1}kinetin. Calli was transferred to solid MS medium containing the same hormones and different concentrations of NaCl (0, 100, 150 mM) and mannitol (0, 180, 275 mM). Obtained calli was incubated under a 16h photoperiod (white fluorescent lamps: irradiance of 58 µmol m^{-2} s^{-1}) and a day/ night temperature of 25±2ºC for 90 days and subcultured every 45 days. Each treatment consisted of 4 replicates (Petri dishes) containing 7 to 10 calli. Calli was assayed after 45 days of culture in terms of some physiological and biochemical parameters.

Proline content

Free proline was determined by the method of Bates et al. (17). Fresh plant material (0.5 g) was homogenized with 10 ml of 3% sulphosalicylic acid and the homogenate was filtrated through filter paper. The resulting solution (1ml) was diluted with 1 ml of distilled water and treated with 2ml acid ninhydrin and 2ml of concentrated glacial acetic acid for 1h at 100ºC and then it cooled rapidly to 0ºC on ice. The reaction mixture was extracted with 4ml toluene, and the absorbance was measured by spectrophotometer (Shimadzu, UV-160, Japan) at 520 nm.

Lipid peroxidation

Malondialdehyde (MDA), which is a secondary end product of polyunsaturated fatty acid oxidation, has been used as an indicator of lipid peroxidation. MDA content was determined by the thiobarbituric acid (TBA) reaction as described by Heath and Packer (18). Fresh plant material (0.5 g) was homogenized in 20% trichloroacetic acid (TCA) containing 0.5% TBA. The mixture was heated at 95ºC for 30 min, quickly cooled in an ice-bath and then centrifuged at 10,000 g for 10 min. The absorbance of supernatant was measured at 532nm, and measurements were corrected for non-specific absorbance at 600nm. The MDA concentration was calculated using 155 mM^{-1} cm^{-1} as extinction coefficient.

Protein isolation and estimation

For protein and enzyme extractions, 1g of fresh plant tissues (seedlings and calli) was homogenized in 3ml of 25mM Tris-HCl buffer (pH 6.8), 3% polyvinylpolypyrrolidone (PVPP) using a mortar and pestle at 4ºC. Homogenate was centrifuged (Heraus centrifuge, D63, Germany) at 4ºC for 1h at 13000g and supernatant was used for enzyme assay and protein determination. Total protein concentrations were measured by the spectrophotometric method of Bradford (19) using bovine serum albumin as the standard.

Antioxidant enzyme activities

The activity of superoxide dismutase (SOD; E.C. 1.15.1.1) was estimated by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium chloride (NBT) described by Giannopolitis and Ries (20). The reaction mixture consisted of 50mM sodium phosphate buffer (pH 7.5), 13 mM L-methionien, 75 µM NBT, 0.1 µM EDTA, 2

µM riboflavin and 50µl enzyme extract. The reaction was started by exposing the mixture to white fluorescent light for 15 min. The reduction in NBT was measured by reading absorbance at 560nm. Blanks and controls were run in same manner but without illumination and supernatant. One unit of SOD activity was defined as the amount of enzyme which causes 50% inhibition of the photochemical reduction of NBT.

Catalase (CAT; E.C. 1.11.1.6) activity was measured according the method of Aebi (21). The reaction mixture contained 2.5ml 50mM sodium phosphate buffer (pH 7.0), 0.3ml H_2O_2 (3%) and 100 µl enzyme extract. The enzyme activity was defined as 1 µmol of H_2O_2 decomposed per min.

The reaction was started by the addition of extract, and decreasing of H_2O_2 was monitored at 240nm for 1min. Enzyme activity was computed by calculating the amount of H_2O_2 decomposed.

Peroxidase (POX; E.C. 1.11.1.7) activity was determined by spectrophotometry method according to Abeles and Biles (22). The reaction mixture contained 4ml of 0.2M acetate buffer (pH 4.8), 0.4ml H_2O_2 (3%), 0.2ml benzidine (20mM) in 50% methanol and 100µl enzyme extract. The increase of absorbance was recorded at 530 nm. The POX activity was defined as 1 µmol of benzidine oxidized per min per mg protein (U mg^{-1}).

Polyphenol oxidase (PPO; E.C. 1.14.18.1) activity was estimated according to the method of Raymond et al. (23). The reaction mixture contained 0.8ml 0.2M sodium phosphate buffer (pH 7.6), 0.02ml of 20mM pyrogallol and 200µl enzyme extract. The increase of absorbance was recorded at 430nm. The temperature of the reaction mixture was 28ºC.

Statistical analyses

Each data point was the average of 4 replicates. Presented data was analyzed by analysis of variance (ANOVA) using SPSS (version 10). The significance of differences was determined according to Duncan's multiple range test (DMRT) at the 0.05 level of probability. Mean values and standard deviation for each treatment are shown in Figures 1-7.

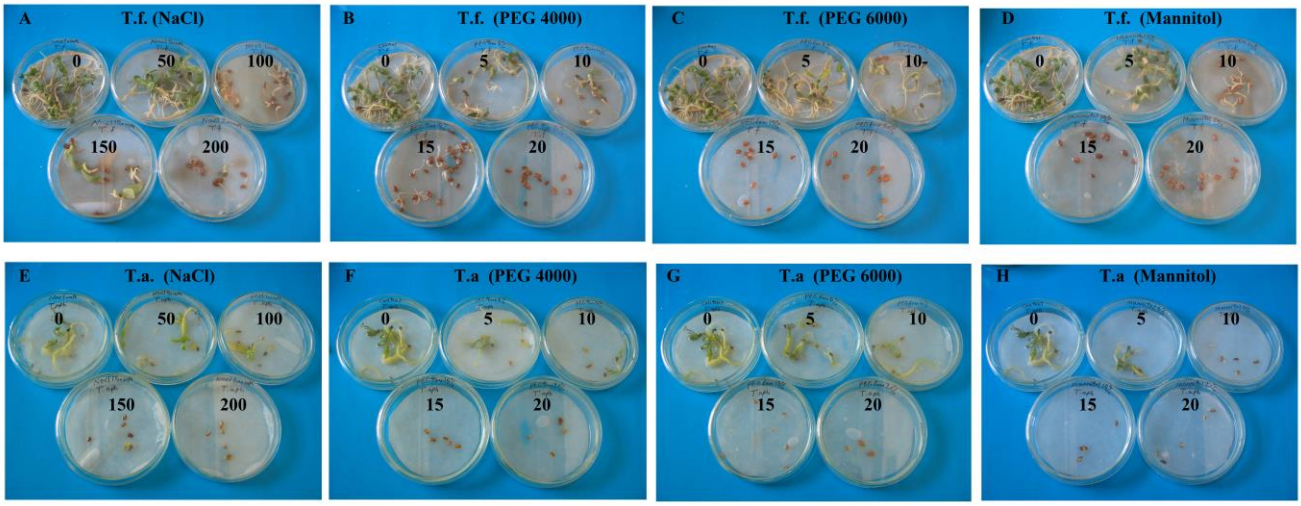

Figure 1. Effects of different concentrations of NaCl (0, 50, 100, 150 and 200 mM NaCl) (A and E), PEG 4000 (0, 5, 10, 15 and 20%) (B and F), PEG 6000 (0, 5, 10, 15 and 20%) (C and G), and mannitol (0, 5, 10, 15 and 20%) (D and H) on seed germination of *Trigonella foenum-graecum* and *T. aphanoneura* after 30 d of treatment

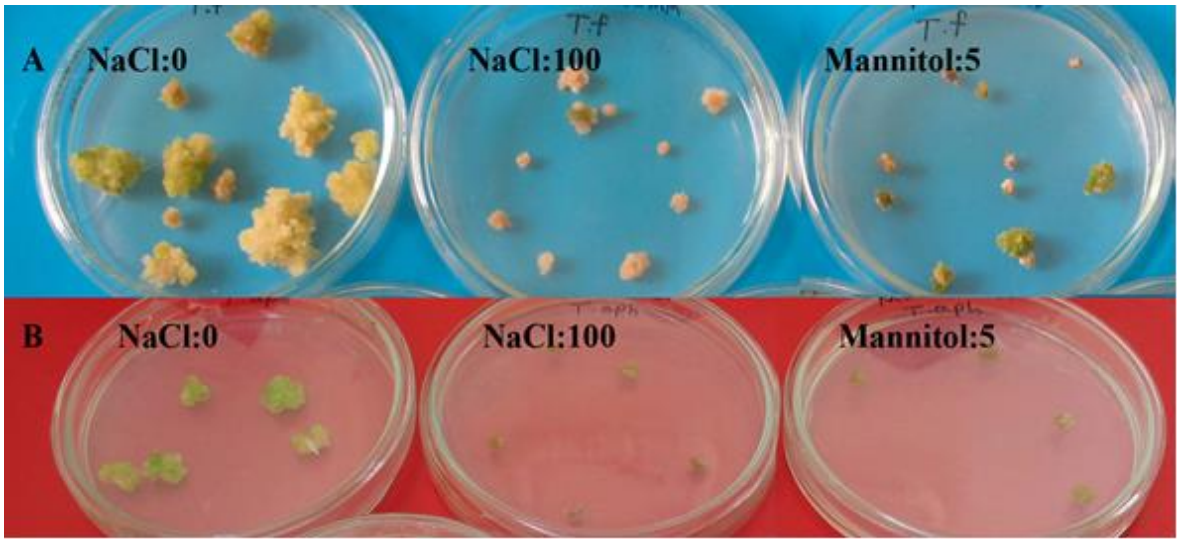

Figure 2. Effects of NaCl (100 mM NaCl), and mannitol (5 %) on callus growth in *T. foenum-graecum* (A) and *T. aphanoneura* (B) after 30 d of explant subculture and treatment

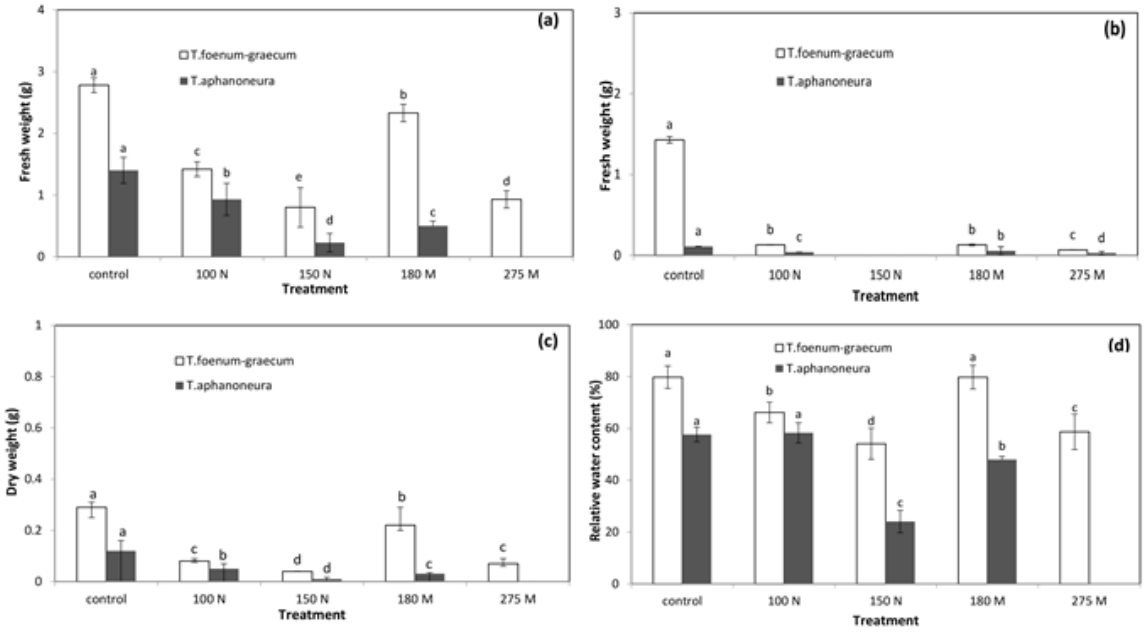

Figure 3. Changes of fresh weight in seedlings (a) and calli (b), dry weight (c) and relative water content (d) in *T. foenum-graecum* and *T. aphanoneura* under salt and drought stresses (N: mM NaCl, M: mM Mannitol). The vertical bars represent standard errors. The mean values marked by the same letter are not significantly different at $P<0.05$ as determined by Duncan's multiple range test.

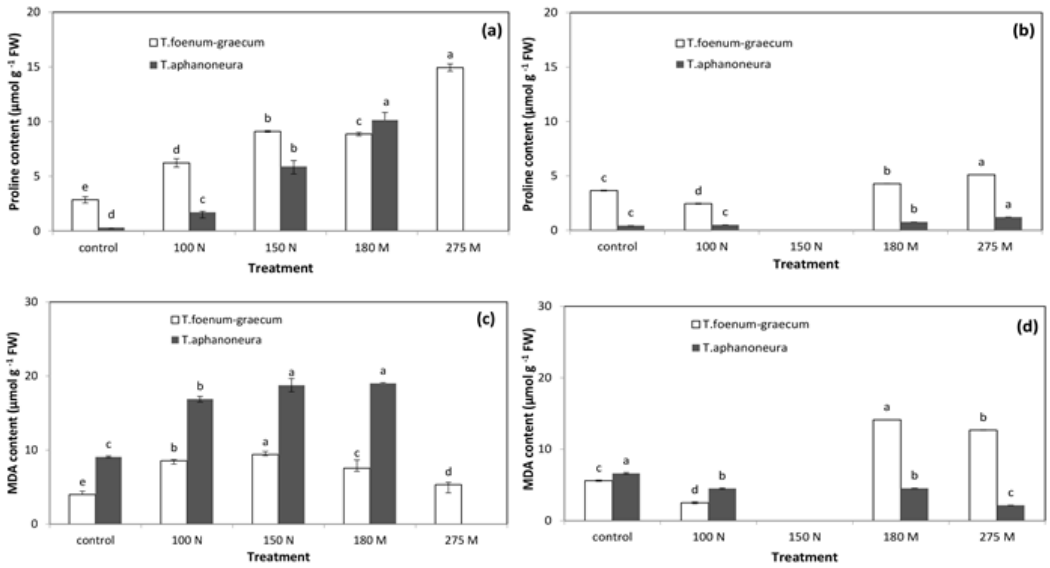

Figure 4. Content of proline (µmol g^{-1} FW) and MDA (µmol g^{-1} FW) in seedlings (a, c) and calli (b, d) of *T. foenum-graecum* and *T. aphanoneura* under salt and drought stresses (N: mM NaCl, M: mM Mannitol). The vertical bars represent standard errors. The mean values marked by the same letter are not significantly different at *P*<0.05 as determined by Duncan's multiple range test.

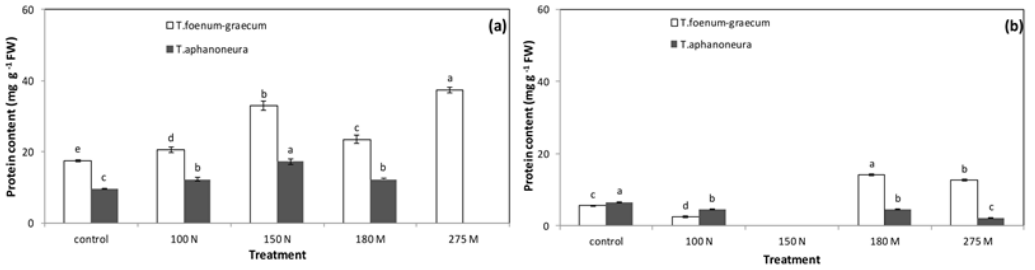

Figure 5. Changes in content of proteins in seedlings (a) and calli (b) of *T. foenum-graecum* and *T. aphanoneura* under salt and drought stresses (N: mM NaCl, M: mM Mannitol)

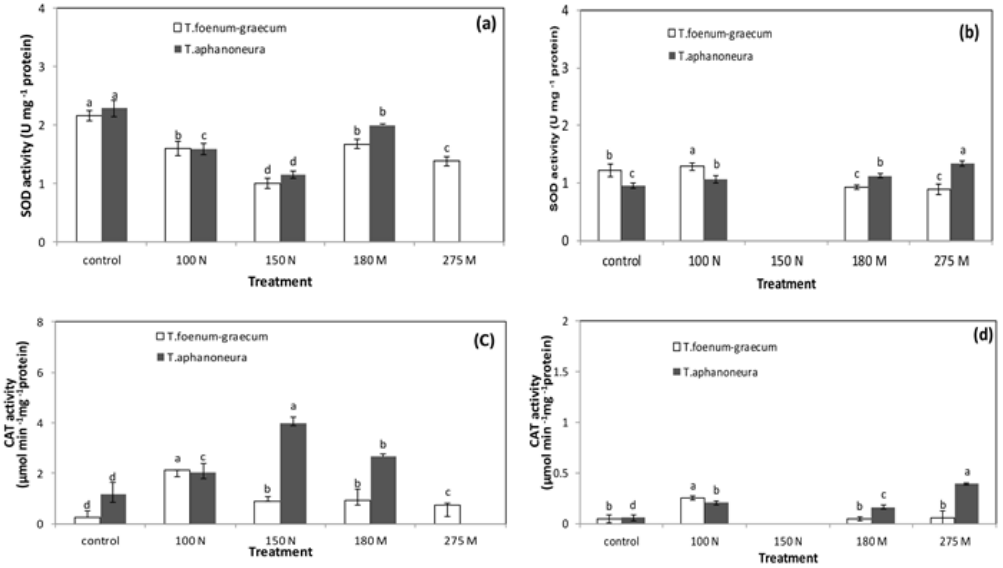

Figure 6. Activity of SOD [U mg $^{-1}$ (protein)] and CAT (µmol $min^{-1}mg^{-1}$ protein) in seedlings (a, c) and calli (b, d) of *T. foenum-graecum* and *T. aphanoneura* under salt and drought stresses (N: mM NaCl, M: mM Mannitol). The vertical bars represent standard errors. The mean values marked by the same letter are not significantly different at *P*<0.05 as determined by Duncan's multiple range test.

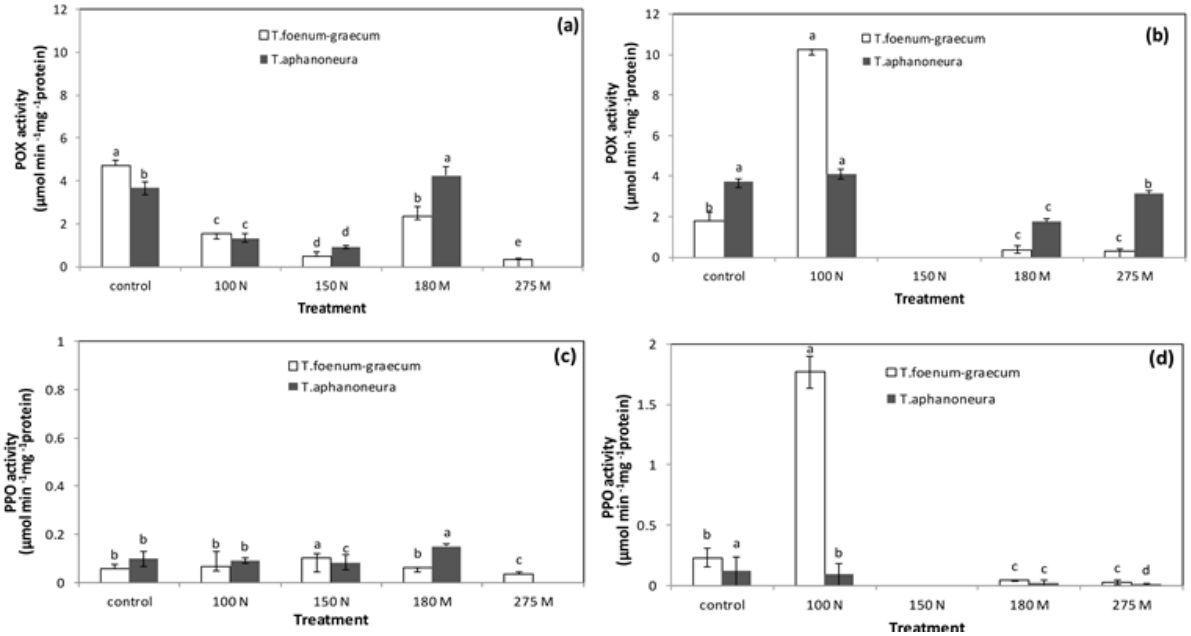

Figure 7. Activity of POX (µmol min^{-1}mg^{-1} protein) and PPO (µmol min^{-1}mg^{-1} protein) in seedlings (a, c) and calli (b, d) of *T. foenum-graecum* and *T. aphanoneura* under salt and drought stresses (N: mM NaCl, M: mM Mannitol). The vertical bars represent standard errors. The mean values marked by the same letter are not significantly different at *P*<0.05 as determined by Duncan's multiple range test.

Results

Seed germination and growth

The growth of *T. foenum-graecum* and *T. aphanoneura* seedlings and calli declined gradually under increasing concentrations of NaCl, PEG and mannitol (Figs. 1, 2, 3a-c). Mannitol caused a significant reduction in *T. aphanoneura* germination, so that at 275mM mannitol approximately no germination was observed (Fig. 3a, c).

Callus induction

The calli was induced from seeds of *T. foenum-graecum* and *T. aphanoneura.* They were cultured on MS medium supplemented with 1 mg l^{-1} 2, 4-D and 0.5 mg l^{-1}kinetin, 3% sucrose and 0.8% agar-agar, pH 5.8. Induced calli was transferred to the same solid MS medium containing different concentrations of NaCl and mannitol. In contrast with PEG, mannitol did not interfere in solidifying the medium. Therefore, mannitol was used here or more commonly for water-deficit stress treatment *in vitro*. Increasing NaCl and mannitol concentrations caused a visible decrease in the growth of callus of both species (Figs. 2 and 3b). Slight growth retardation and sensible necrosis was observed in the calli grown at 100 mM NaCl, and 180 and 275 mM mannitol.

Growth

A significant decline was observed in fresh weight of both species seedlings under NaCl. The decrease in fresh weight of *T. aphanoneura* (83.6 %) was significantly more than that of *T. foenum-graecum* (71%) at 150 mM NaCl (Fig. 3). Moreover, the decrease in fresh weight of *T. foenum-graecum* under iso-osmotic concentration of salinity (-71% at 150 mM NaCl) was greater than that of mannitol (-66.5% at 275 M mannitol). None of the calli was able to growth at 150 mM

NaCl. Growth of the callus was gradually decreased as the concentration of NaCl, and mannitol increased (Fig. 3b). The growth and germination of *T. foenum-graecum* under NaCl and mannitol was also more than that of *T. aphanoneura* (Fig. 1).

Changes of relative water content

NaCl and mannitol decreased RWC significantly in seedlings of both *Trigonella* species at 150 mM NaCl and 275 mM mannitol (Fig. 3d). According to growth parameters, the RWC decrease in *T. aphanoneura* seedlings (-58.2%) was higher than that of *T. foenum-graecum* (32.2 %) at 150 mM NaCl. The decrease of RWC in *T. foenum-graecum* under 275 mM mannitol (-26.4%) was lower than that of 150 mM NaCl (-32.2%).

Proline content

The results of our study revealed that all NaCl and mannitol concentrations significantly increase proline content in seedlings of both *Trigonella* species. The increase in proline content in calli was observed only under mannitol treatment. *Trigonella foenum-graecum* contained more proline than *T. aphanoneura* under both NaCl and mannitol induced stresses (Figs. 4a, b). However, the rate of increase of proline content in seedlings of *T. aphanoneura* (+1768%) was higher than that of *T. foenum-graecum* (+219.7%) at 150 mM NaCl. Contrary to that of seedlings, proline content was slightly reduced in callus of fenugreek under 100 mM NaCl and did not change significantly in callus of *T. aphanoneura* (Fig. 4b). Moreover, mannitol induced more proline in seedlings of *T. foenum-graecum* comparing to that of NaCl.

Lipid peroxidation

MDA content in seedlings of *T. aphanoneura* was higher than that of *T. foenum-graecum* under control condition and increased in both species under salinity (Fig. 4c). Contrary to that of proline content, the percentage of increase in MDA content in *T. aphanoneura* was lower than that of *T. foenum-graecum*. However, absolute content of MDA in *T. aphanoneura* at all conditions was higher than that of *T. foenum-graecum*. Moreover, MDA content in seedlings and calli of *T. foenum-graecum* under mannitol stress was higher than that of control.

Under salinity stress, seedlings of *T. foenum-graecum* showed a lower MDA content than that of *T. aphanoneura* (Fig. 4c). Contrary to that of the seedlings, MDA content in calli of *T. aphanoneura* was significantly lower than that of *T. foenum-graecum* under mannitol treatments. Moreover, MDA content in calli of *T. foenum-graecum* under mannitol stress is higher compared to that of seedlings.

Total soluble protein content

Salt stress caused an increase in protein content in seedlings of both species. Inducing effect of mannitol on protein content in seedling of both species was also observed (Fig. 5a). Salinity decreased the protein content in callus of both species (Fig. 5b). In *T. foenum-graecum*, protein content of calli was decreased at 100 mM NaCl in comparison with control. In contrast, the protein content was increased by 180mM mannitol, and decreased in 275mM mannitol (Fig. 5b). Therefore, the level of protein content at 180 mM and 275 mM mannitol in seedlings and calli of *T. foenum-graecum* was higher than that of NaCl stress.

Superoxide dismutase activity

A considerable and significant decrease in the activity of SOD was recorded in seedlings of two *Trigonella* species under NaCl and mannitol treatment (Fig. 6a). SOD activity in concentrations of 275mM mannitol was higher than that of 150mM NaCl in seedlings of *T. foenum-graecum*. As shown in Figure 6b, NaCl and mannitol increased the activity of SOD in calli of *T. aphanoneura*. We also observed that in the callus of *T. foenum-graecum*, NaCl resulted in higher SOD activity as compared to the control, while 180mM mannitol significantly decreased SOD activity. Meanwhile, SOD activity at 275mM mannitol did not have significant difference with 180 mM mannitol in callus of *T. foenum-graecum* (Fig 6b).

Catalase activity

The results indicated that catalase (CAT) activity increased in seedlings of *T. foenum-graecum* when salinity increased up to 100 mM NaCl, then decreased with increasing the salinity to 150mM which is not lower than that of control yet. Mannitol also caused significant enhancement in activity of CAT in seedlings of *T. foenum-graecum* compared to control. However, CAT activity increased steadily in seedlings of *T. aphanoneura* under NaCl (Fig. 6c). CAT activities in calli under all treatments were lower than that of seedlings in both species. Moreover, in calli of *T. aphanoneura*, CAT activity at 275 mM mannitol was higher than that of all other treatments. In calli of *T. foenum-graecum* no significant changes were observed in activity of CAT under mannitol stress (Fig. 6d).

Peroxidase activity

Peroxidase (POX) activities of the seedlings and calli of two *Trigonella* species are shown in Figures 7a-b. Seedlings of *T. foenum-graecum* and *T. aphanoneura* showed a significant and continued decline in POX activity with increasing concentrations of NaCl and mannitol (Fig. 7a). Conversely, as shown in Figure 7b, POX activity was increased in calli of both species at 100mM NaCl but decreased at 180 and 275mM mannitol comparing to that of control.

Polyphenol oxidase Activity

NaCl and mannitol caused no prominent trend in PPO activity in seedlings and calli of two *Trigonella* species (Figs. 7c-d). The only mentionable difference in PPO activity between seedlings and calli is slightly higher level of PPO in calli comparing to that of seedlings. NaCl at 100mM increased the PPO activity in calli of *T. foenum-graecum*. No significant change was observed in activity of PPO in calli of *T. aphanoneura* (Fig. 7d).

Discussion

The present work carried out for comparing the effects of salinity and drought on growth and biochemistry of seedlings and calli in two species of *Trigonella*. According to our knowledge, no published data was shown for *T. aphanoneura* under mannitol stress. Similarly, the results of Niknam et al. (24) and Ghorbanpour et al. (25) indicated significant decrease in seed germination in fenugreek under salinity and drought stresses, respectively.

In vitro cultures including tissue culture have been used as suitable tools for study, selection, characterization and production of tolerant variants against salt stress (9, 24, 26, 27). Growth retardation was observed in the calli grown under NaCl and mannitol stress. Similarly, the studies of Niknam et al. (28) revealed that growth of calli in

Acanthophyllum glandulosum Bunge ex. Boiss. and *Acanthophyllum sordidum* Bunge ex. Boiss. decreased with increasing salinity. Soheilikhah et al. (29) reported also a significant decrease of growth and water content observed in callus of *Carthamus tinctorius* L. under NaCl and mannitol.

Both of the salinity and drought treatments led to significant decline in growth of seedlings and calli of both *Trigonella* species. The differences in growth and water content under salt and drought stress could be due to the ionic effects of NaCl comparing to mannitol. NaCl and mannitol imposed greater effects on growth of calli in both species compared to control. Aghaleh et al. (6) also reported the decrease in growth parameters of *Salicornia* seedlings at high salinity levels (100 to 200mM NaCl).

RWC is the best growth indices revealing the stress intensity (30). Loss of turgor can be determined by decreasing of RWC in plants that resulted in declining of water availability for cell extension processes (31). The differences in RWC could also be due to the deleterious ionic effects of NaCl comparing to mannitol. Difference between two species regarding RWC content could be due to different osmotic adjustment capacity of the species.

Proline as an osmoticum normally accumulates in large quantities to maintain membrane structure and acts as free radical scavengers preventing lipid peroxidation in response to osmotic stresses (32, 33). Also, proline seemed to improve salt tolerance by protecting some antioxidative enzyme activities, photosynthetic activity and finally maintaining of plant growth and water status (34). Yang et al. (35) argued that high levels of proline accumulation in plants may be known as a symptom of stress. In another study, Shobbar et al. (36) determined that NaCl concentrations significantly increase proline content in all cultivars of rice. Recently, Karimi et al. (37) showed that proline content increased by an enhancement in both NaCl and mannitol concentrations in the calli of *Carthamus tinctorius*.

The oxidative damage has been evaluated here as malondialdehyde (MDA) content which is a product of lipid peroxidation. Determination of lipid peroxidation has often been used as a tool to assess the degree of plant sensitivity to oxidative damage (38). The increased level of lipid peroxidation which can be considered as a stress indicator was observed under prolonged drought stress in many plants such as bentgrass (39). Also, these results are in agreement with the results of Torabi and Niknam (40), who reported increased MDA content under salt and drought stresses in *Salicornia europaea* L. In calli of *Salicornia europaea* and *Salicornia persica* Akhani the level of lipid peroxidation followed a different pattern under salt and drought stress (40). According to Niknam et al. (28) callus of *Acanthophyllum glandulosum* showed a lower MDA content than that of *A. sordidum* under salinity stress.

Seedlings of *T. foenum-graecum* showed a lower MDA content than that of *T. aphanoneura*. This indicates that the seedlings of *T. foenum-graecum* are able to tolerate salinity-induced oxidative damage better than that of *T. aphanoneura*. This tolerance might be a result of the significantly higher activities of antioxidant enzymes or higher proline content in the seedlings of *T. foenum-graecum.* However, MDA content in seedling of *T. foenum-graecum* under mannitol stress is higher compared to that of iso-osmotic NaCl concentrations. According to the MDA analyses, calli responded differently to the salinity and drought compared to that of seedlings.

Changes in protein expression, accumulation, and synthesis have been observed in plants on exposure to salt stress (22). Environmental stresses bring quantitative as well as qualitative changes in proteins. Stress induced protein accumulation may provide a storage form of nitrogen and is used by the plant later and has been proved to play a role in osmotic adjustment. In accordance to our results, increase in protein content could be due to synthesis of new stress proteins or antioxidative enzymes which is in accordance with the increase in the activity of CAT. Shobbar et al. (36) also reported that total protein content in seedlings of the *Oryza sativa* was raised under both of salinity and drought. The increase in total protein in response to stress contrasts the recent findings for *in vitro* grown cucumber (7). The decrease in protein content which may also was observed in our research at higher salinities in seedlings or in calli may be because of the release of some protein to the media due to osmotic shock (41) or a decrease in the synthesis (42) and an increase in the hydrolysis of protein (43). This result was consistent with the findings of Ge et al. (44) in maize.

Superoxide dismutase (SOD) as an antioxidant system plays a key role in quenching active oxygen (45), working as catalyzing the dismutation of superoxide radical to molecular oxygen and H_2O_2 which are eliminated by CAT, POX and other antioxidant enzymes. Variable responses of SOD to water deficit were reported in literature; for example a significant decrease in bentgrass (39), unaffected in cowpea leaves (46) and increased in rice (35). SOD cannot be considered solely responsible for protection against peroxidation because it converts superoxide radical anion (O_2^-) to H_2O_2 which is also a ROS. This ROS should be then scavenged by other enzymes, such as CAT and POX. Catalase plays an important role in the removal of H_2O_2 level in plants (3). Munir and Aftab (47) indicated that catalase activity of sugarcane cultivars showed an increasing trend under salt stress. Our findings contradict with previous works where decreased catalase activity was observed in canola cultivars under drought stress (48). Difference in salt tolerance and biochemical activities among plant species could be due to the nature of specific stress and their ability in producing osmoprotectants such as proline. Changcheng et al. (49) found that severe drought stress largely decreased the activities of antioxidant enzymes in *Cinnamomum bodinieri* H. Lév., *Platycarya longipes* Wu and *Pteroceltis tatarinowii* Maxim. On the other hand, scavenging function of antioxidant enzymes such as POX was impaired by severe stress (46). Moreover, Ben Amor et al. (50) described that peroxidase activity in the *Cakile maritima* Scop. increased gradually with increasing NaCl concentrations up to 400 mmol l^{-1}, whereas POX started to decrease in plants treated with 400 mmol l^{-1} NaCl. Polyphenol oxidase (PPO) is the major enzyme responsible for oxidation of phenolic compounds. In agreement to our results, increased PPO activity was also reported by Demir and Kocaliskan (51) in bean seedlings.

Conclusion

The present study reveals substantial differences in seedlings and calli of *T. foenum-graecum* and *T. aphanoneura* in regard to NaCl and mannitol stresses. Exposure of salt and drought resulted in reduction of fresh and dry weight, relative water content, alteration of proline and protein content as well as antioxidant activities in both species of *Trigonella in*

vitro. The degree of stress was assessed according to Hsiao (52), in which a loss of 8%–10% RWC was defined as a mild stress, a loss of 10%–20% as moderate stress and above of 20% as severe stress. In our study, comparing of iso-osmotic concentrations showed that 150mM NaCl and 275mM mannitol produced severe stress in both species. While, iso-osmotic concentrations of 100mM NaCl and 180mM mannitol induced moderate and mild stress in *T. foenum-graecum* and mild and severe stress in *T. aphanoneura*, respectively.

In seedlings of two *Trigonella* species ROS scavenging antioxidant system such as SOD, POX and PPO seems to be inadequate; therefore, other resistance mechanisms such as accumulation of proline and protein are necessary to cope with salt and drought stress. In our study, non-enzymatic antioxidative compounds could be effective in scavenging ROS in seedlings of *T. foenum-graecum*. Higher accumulation of MDA in seedlings of *T. aphanoneura* was the result of the lower protective mechanisms in these species in comparison with *T. foenum-graecum.*

The lower values of MDA in calli of both species under salt stress indicate that at the cellular level these species are equipped with efficient free radical scavenging systems that offers protection against oxidative stress. Induction in the activities of CAT and POX in calli of both species could verify the above mentioned protection mechanisms. On the other hand, the level of lipid peroxidation in both species decreased under mannitol stress and these decreases are in accordance with increase in activities of SOD, CAT and POX.

According to this study, a lower lipid peroxidation combined with higher amount of proline and protein could possibly explain the ability of *T. foenum-graecum* seedlings to grow at higher NaCl concentrations than *T. aphanoneura*, which appears to be more salt-sensitive. This study provides a comparative understanding of the responses of *T. foenum-graecum* and *T. aphanoneura* and calli seedlings to culture in saline and drought conditions, which is important for future studies aimed at developing strategies for selecting salt-tolerant plants.

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