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# Effect of NaCl on biomass, protein and proline contents, and antioxidant enzymes in seedlings and calli of two *Trigonella* species

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## Abstract

The effects of NaCl on growth, contents of proteins and proline, and activities of catalase, peroxidase and polyphenol oxidase were investigated in seedlings and calli of *Trigonella foenum-graecum* L. and *T. aphanoneura* Rech. f. Seeds and hypocotyl explants were cultured on Murashige and Skoog medium supplemented with 0, 50, 100, 150 and 200 mM NaCl. Seed germination and the fresh and dry mass of the seedlings decreased significantly under salinity. In both species significant increases in protein content of seedlings over that of control were observed at 150 and 200 mM NaCl. Protein content in calli decreased at 200 mM NaCl over that of control. Protein content was higher in seedlings than in calli at all NaCl concentrations. Conversely, proline content was lower in seedlings than in calli at all the tested NaCl concentrations. NaCl caused changes in the activities of peroxidase, catalase and polyphenol oxidase in seedlings and calli.

*Additional key words*: catalase, *in vitro* culture, peroxidase, polyphenol oxidase, salinity.

## Introduction

Salinity limits the production of nearly 40 % of agricultural lands the world over (Serrano and Gaxiola 1994). Salinity affects numerous physiological or biochemical processes, many of which are seen at the cellular level. *In vitro* culture techniques provide controlled, uniform environments to study the salt-stress response of undifferentiated callus, thus eliminating complications arising from genetic and morphological variability associated with tissues of whole plants even within the same species (McCoy 1987). Callus culture of date palm subjected to salt stress exhibited reduction in growth, accumulation of proline, and increase in sodium and decrease in potassium ion concentrations (Al-Khayri 2002). The studies on metabolic effects of NaCl salinity on callus cultures have been received much attention in recent years (*e.g*. Olmos and Hellin 1996, Al-Khayri 2002, Cherian and Reddy 2003, Niknam *et al*. 2004).

Salinity also induce oxidative stress in plants (Holmberg and Bulow 1998, Bartosz 1997). Hence, the salt-tolerant plants besides being able to regulate the ion and water movements should also have an efficient antioxidative system for effective removal of the reactive oxygen species (ROS) (Rout and Shaw 2001). Plants protect cells and subcellular systems from the effects of ROS by enzymes such as superoxide dismutase (SOD), catalase, peroxidase, glutathione reductase, polyphenol oxidase and non-enzymic antioxidants such as ascorbate and glutathione (Agarwal and Pandey 2004).

*Trigonella* L. is a large genus with close to 135 species belonging to the family *Leguminosae* (*Fabaceae*). Most of the species are distributed in the dry regions (Townsend 1974). The objective of this work was to investigate the merit of using *in vitro* cultures, such as seedlings and calli to provide a system for studying salinity induced changes in content of proline and protein and antioxidant enzymes in two *Trigonella* species.

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*Abbreviations*: CAT - catalase; 2,4-D - 2,4-dichlorophenoxy acetic acid; Kin - kinetin; POX - peroxidase; MS medium - Murashige and Skoog (1962) medium; PPO - polyphenol oxidase; ROS - reactive oxygen species; SDS-PAGE - sodium dodecyl-sulfate polyacrylamid gel electrophoresis.

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## Materials and methods

*Trigonella foenum-graecum* L. and *T. aphanoneura* Rech. f. seeds were surface sterilized with 10 % sodium hypochlorite solution and then washed several times with sterile distilled water. Seeds were germinated and maintained on Murashige and Skoog (MS) media solidified with 8 % agar, 30 g $dm^{-3}$ sucrose and containing 0, 50, 100, 150 and 200 mM NaCl under 16-h photoperiod (white fluorescent lamps; irradiance of 46 μmol $m^{-2}$ $s^{-1}$) and day/night temperature of 25/20 °C. Calli were established from surface-sterilized hypocotyl explants obtained from 7-d-old seedlings on MS medium, supplemented with 1 mg $dm^{-3}$ 2,4-dichloro-phenoxy acetic acid (2,4-D) and 0.5 mg $dm^{-3}$ kinetin (Kin). Seedlings and hypocotyl explants were maintained in a growth chamber for 28 and 60 d, respectively. Fresh mass and dry mass of seedlings and calli were recorded in 15- and 60-d-old samples. Three replicates containing 5 seedlings and 6 calli each were taken for measurements. Dry mass was determined after drying the sample at 60 °C for 48 h to constant mass.

Free proline was determined by the method of Bates *et al.* (1973). Approximately 0.5 g of plant material was homogenized for 5 min in 5 $cm^3$ of 3 % aqueous sulphosalicylic acid. Two $cm^3$ of the extract reacted with 2 $cm^3$ of glacial acetic acid for 1 h at 100 °C. The reaction mixture was extracted with 4 $cm^3$ toluene and the absorbance was read at 520 nm.

For estimation of total protein content and enzyme activity, 1 g each fresh seedling and callus tissue was homogenized in 5 $cm^3$ of 1 M Tris-HCl (pH 6.8). The homogenate was filtered through 4 layers of cheescloth and centrifuged at 12 100 *g* for 1 h at 4 °C and the supernatant was used for enzyme assays and protein determination. Total protein concentrations were measured by the spectrophotometric method of Lowry using bovine serum albumin as the standard (Lowry *et al.* 1951).

Peroxidase (POX; E.C. 1.11.1.7) activity was measured according to the method of Abeles and Biles (1999). The assay mixture consisted of 4 $cm^3$ of 0.2 M acetate buffer (pH 4.8), 0.4 $cm^3$ $H_2O_2$ (3 %), 0.2 $cm^3$ 20 mM benzidine and 0.05 $cm^3$ enzyme extract. The absorbance was recorded at 530 nm.

Catalase (CAT; E.C. 1.11.1.6) activity was assayed from the rate of $H_2O_2$ decomposition as measured by decrease by the decrease of absorbance at 240 nm, following the procedure of Aebi (1974). The reaction mixture contained 2.5 $cm^3$ 50 mM sodium phosphate buffer (pH 7.0), 0.3 $cm^3$ $H_2O_2$ (3 %), and 0.2 $cm^3$ enzyme extract.

Polyphenol oxidase (PPO; E.C. 1.10.3.1) activity was estimated following the method of Raymond *et al.* (1993) by measuring the increase in absorbance at 430 nm. The reaction mixture contained 2.5 $cm^3$ 200 mM sodium phosphate buffer (pH 6.8), 0.2 $cm^3$ pyrogallol 20 mM and 0.1 $cm^3$ enzyme extract. The temperature of the reaction mixture was 40 °C.

Discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970) with 12 % acrylamide gels. For detection of proteins, gels were stained with 0.03 % Coomassie Briliant Blue G250. Native gel electrophoresis (non-denaturing conditions) for isoenzymes assay was carried out according to a modified method of Davis (1964) with a 10 % acrylamide gel at 4 °C. A vertical electrophoresis apparatus (*model LKB*, Bromma, Sweden) was used. The electrophoretic run was carried out with 120 mV (30 mA) per plate towards the cathode. Electrophoretic pattern of POX was obtained by staining the gels by benzidine according to Van Loon (1971). The gels immersed in 0.2 M acetate buffer (pH 4.8) containing 3 % $H_2O_2$ and 4 % benzidine in 50 % methanol at room temperature till the brown color. CAT activity was determined as described by Woodburry *et al.* (1971). Gels were incubated in 0.01 % $H_2O_2$ for 10 min and developed in a 0.5 % (m/v) $FeCl_3$ and 0.5 % $K_3Fe(CN_6)$ (m/v) solution for 10 min. A high-speed centrifuge (*J2-21M*, *Beckman*, Palo Alto, USA) and UV-visible recording spectrophotometer (*UV-160*, *Shimadzu*, Tokyo, Japan) with 10 mm matched quartz cells were used for centrifugation of the extracts and determination of the absorbance, respectively.

The data determined in triplicate were analyzed by analysis of variance (*ANOVA*) using *SPSS* (version *9.05*). The significance of differences was determined according to Duncan's multiple range test (DMRT). $P$ values < 0.05 are considered to be significant.

## Results

Fresh and dry mass of seedlings of *T. foenum-graecum* decreased at 50 mM NaCl but increased at 100 mM and then decreased at higher salinities. In contrast, fresh mass of calli decreased at all salinities. Dry mass of calli was not determined. The salinity up to 100 mM reduced free proline content of seedlings, 150 mM NaCl returned it to nearly that of the control and 200 mM NaCl increased it significantly.

NaCl decreased the proline content of calli in respect to that of control. NaCl at 150 mM and higher increased the protein content of seedlings significantly. Contrary to that of seedlings, NaCl at 100 mM and more reduced

Table 1. Fresh and dry mass [mg] and the content of free proline [μg $g^{-1}$(f.m.)] and proteins [mg $g^{-1}$(f.m.)], and activities of POX [$\Delta A_{530}$ $g^{-1}$(f.m.) $min^{-1}$], CAT [$\Delta A_{240}$ $g^{-1}$(f.m.) $min^{-1}$] and PPO [$\Delta A_{430}$ $g^{-1}$(f.m.) $min^{-1}$] in seedlings (S) and calli (C) of *T. foenum-graecum* L. under NaCl [mM] stress. Values are means ± SE of 3 determinations.

| NaCl | | Fresh mass | Dry mass | Proline | Proteins | POX | CAT | PPO |
|---|---|---|---|---|---|---|---|---|
| 0 | S | 176 ± 42 | 30 ± 2.7 | 103 ± 6.0 | 11.7 ± 0.04 | 20.44 ± 1.28 | 1.86 ± 0.07 | 6.06 ± 0.77 |
| | C | 230 ± 78 | - | 501 ± 12.0 | 9.3 ± 0.07 | 16.27 ± 6.00 | 0.31 ± 0.05 | 3.37 ± 0.15 |
| 50 | S | 98 ± 6 | 19 ± 10.4 | 62 ± 5.6 | 13.3 ± 0.04 | 47.97 ± 0.04 | 5.14 ± 0.16 | 7.29 ± 0.88 |
| | C | 106 ± 21 | - | 226 ± 1.5 | 9.3 ± 0.04 | 33.17 ± 0.52 | 0.43 ± 0.42 | 5.59 ± 0.99 |
| 100 | S | 110 ± 26 | 11 ± 2.3 | 65 ± 7.0 | 12.0 ± 0.03 | 48.20 ± 3.16 | 7.15 ± 0.22 | 7.37 ± 0.30 |
| | C | 53 ± 6 | - | 293 ± 1.7 | 8.8 ± 0.02 | 41.27 ± 3.04 | 1.78 ± 0.10 | 7.95 ± 1.84 |
| 150 | S | 95 ± 14 | 12 ± 0.0 | 98 ± 11.0 | 14.5 ± 0.06 | 15.61 ± 0.34 | 14.40 ± 2.17 | 6.73 ± 0.40 |
| | C | 45 ± 35 | - | - | 7.2 ± 0.02 | 50.24 ± 0.39 | 0.52 ± 0.02 | 26.89 ± 1.98 |
| 200 | S | 77 ± 36 | 12 ± 2.7 | 420 ± 18.0 | 19.7 ± 0.03 | 6.98 ± 1.51 | 13.30 ± 0.40 | - |
| | C | 10 ± 5 | - | - | 4.7 ± 0.04 | 45.23 ± 0.16 | 0.13 ± 0.10 | 32.56 ± 1.16 |

slightly protein content in calli (Table 1).

According to the SDS-PAGE analysis of *T. foenum-graecum* proteins (Fig. 1) the banding pattern of seeds is different from those of seedlings. The intensity of seedling protein bands increased at 150 and 200 mM NaCl significantly. Moreover, NaCl induced also qualitative changes in proteins of seedlings and some new bands appeared at 150 and 200 mM NaCl.

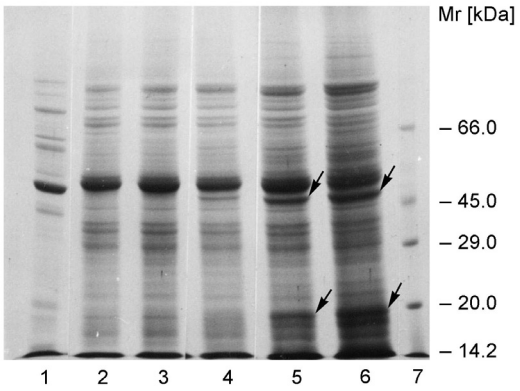


Fig. 1. SDS-PAGE pattern of proteins in seeds and seedlings of *T. foenum-graecum*: seed (1), seedlings at 0, 50, 100, 150 and 200 mM NaCl (2 to 6), and molecular mass marker (7). *Arrows* indicate the new bands in seedlings.

POX activity in seedlings of *T. foenum-graecum* increased significantly up to 100 mM NaCl, but at higher salinities decreased to values lower than that of control. In contrast, POX activity in calli increased significantly up to 150 mM NaCl and the activity at 200 mM NaCl was slightly lower than that of the 150 mM NaCl (Table 1). CAT activity in seedlings increased significantly up to 150 mM NaCl, but that of calli increased up to 100 mM, and at higher salinity decreased. NaCl did not induced any significant changes in the activity of PPO in seedlings, but 150 and 200 mM NaCl increased the PPO activity in calli significantly.

According to PAGE analysis of POX in seedlings, NaCl induced both quantitative and qualitative changes. At 50 and 100 mM NaCl, the specific isoenzyme bands of seeds were disappeared completely in seedlings and new bands specific for seedlings appeared. At higher salinities, the intensity of specific isoenzyme bands of seedlings decreased and some bands appeared likes those of seeds (Fig. 2).

Fresh mass of *T. aphanoneura* seedlings decreased at all salinities. But dry mass of seedlings decreased at 50 mM NaCl but increased at 100 mM and then decreased at higher salinities. In contrast, fresh mass of calli decreased at 50 mM NaCl, remained unchanged at 100 mM and then decreased at higher salinities.

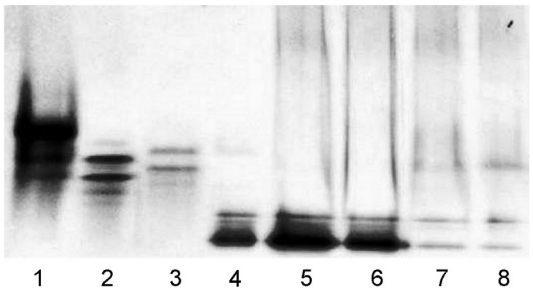


Fig. 2. Activity staining for POX in seeds of *Trigonella* species and seedlings of *T. foenum-graecum* under different concentrations of NaCl: seeds of *T. monantha* (1), seeds of *T. aphanoneura* (2), seeds of *T. foenum-graecum* (3) and seedlings of *T. foenum-graecum* subjected to 0, 50, 100, 150 and 200 mM NaCl (4 to 8).

Free proline content of seedlings increased slightly at the tested salinities (Table 2). In calli, 50 mM NaCl did not changed significantly the proline content in respect to that of control, but further increase in NaCl increased it significantly. The protein content of seedlings increased at all salinities significantly. In contrast, NaCl did not induced any significant changes in protein content of calli.

Table 2. Fresh and dry mass [mg] and the content of free proline [μg $g^{-1}$(f.m.)] and proteins [mg $g^{-1}$(f.m.)], and activities of POX [$\Delta A_{530}$ $g^{-1}$(f.m.) $min^{-1}$], CAT [$\Delta A_{240}$ $g^{-1}$(f.m.) $min^{-1}$] and PPO [$\Delta A_{430}$ $g^{-1}$(f.m.) $min^{-1}$] in seedlings (S) and calli (C) of *T. aphanoneura* under NaCl [mM] stress. Values are means ± SE of 3 determinations.

| NaCl | | Fresh mass | Dry mass | Proline | Proteins | POX | CAT | PPO |
|---|---|---|---|---|---|---|---|---|
| 0 | S | 120 ± 26.0 | 10 ± 1.4 | 132 ± 12.2 | 10.5 ± 0.01 | 101.20 ± 16.4 | 7.33 ± 0.21 | 10.23 ± 1.85 |
| | C | 200 ± 27.0 | - | 406 ± 6.8 | 7.8 ± 0.03 | 33.67 ± 1.4 | 0.20 ± 0.10 | 36.78 ± 1.20 |
| 50 | S | 72 ± 20.0 | 6 ± 1.7 | 178 ± 6.2 | 12.4 ± 0.11 | 83.90 ± 9.9 | 12.49 ± 0.40 | 12.07 ± 0.09 |
| | C | 110 ± 9.8 | - | 420 ± 15.4 | 7.6 ± 0.01 | 31.87 ± 0.0 | 0.91 ± 0.04 | 35.63 ± 2.13 |
| 100 | S | 35 ± 8.0 | 7 ± 3.8 | 220 ± 14.8 | 13.4 ± 0.05 | 51.33 ± 9.8 | 10.81 ± 1.10 | 16.70 ± 0.61 |
| | C | 110 ± 23.0 | - | 534 ± 15.6 | 8.1 ± 0.05 | 30.97 ± 1.6 | 5.69 ± 0.24 | 43.47 ± 1.29 |
| 150 | S | 30 ± 0.5 | 5 ± 0.4 | - | 15.5 ± 0.04 | 62.01 ± 6.6 | 11.73 ± 0.44 | 16.35 ± 1.12 |
| | C | 63 ± 9.1 | - | 550 ± 30.0 | 8.0 ± 0.05 | 43.72 ± 1.9 | 9.14 ± 0.17 | 24.05 ± 4.90 |
| 200 | S | 15 ± 1.9 | 4 ± 0.2 | - | 29.0 ± 0.06 | 15.65 ± 0.2 | 2.11 ± 0.47 | 30.19 ± 0.55 |
| | C | 33 ± 7.5 | | - | 6.5 ± 0.01 | 33.44 ± 30.0 | 10.96 ± 0.24 | 15.04 ± 0.10 |

According to the SDS-PAGE, banding patterns of seed proteins of *T. aphanoneura* is different from those of seedlings (Fig. 3). The intensity of seedling protein bands increased at all salinities. Moreover, qualitative changes in protein were also induced by NaCl and about two prominent new bands (Mr 15 - 16 kDa) appeared at 100 to 200 mM NaCl. These two bands which are present in seeds, are absent in seedlings at control and 50 mM NaCl and 100 mM NaCl and more induced them again.

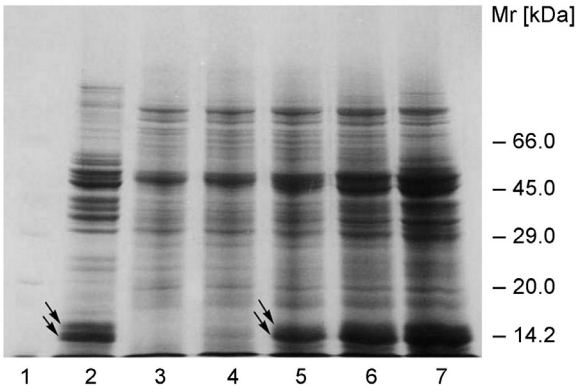


Fig. 3. SDS-PAGE pattern of proteins in seeds and seedlings of *T. aphanoneura* under different concentrations of NaCl: Molecular mass markers (1), seed (2) and seedlings at 0, 50, 100, 150 and 200 mM NaCl, (3 to 7). *Arrows* indicate the affected protein bands.

POX activity in seedlings of *T. aphanoneura* decreased up to 100 mM NaCl, then increased at 150 mM NaCl and at 200 mM NaCl decreased to lower than that of control. In contrast, POX activity in calli decreased slightly up to 100 mM NaCl and then increased at 150 mM and returned to that of control at 200 mM NaCl (Table 1). CAT activity in seedlings increased up to 150 mM NaCl, but at 200 mM NaCl decreased significantly to lower than that of control. CAT activity in calli which was much lower than that of seedlings, increased slightly at 50 mM NaCl but then increased significantly at higher concentrations of NaCl. PPO activity in seedlings increased up to 100 mM NaCl, and remained unchanged at 150 mM NaCl and then increased significantly at 200 mM NaCl. NaCl at 50 mM did not changed PPO activity in calli, 100 mM NaCl increased PPO activity and at higher concentrations of salinity the activities decreased to lower than that of control.

NaCl induced both quantitative and qualitative changes in POX in seedlings of *T. aphanoneura*. At control, in addition to specific POX bands of seedlings, a band likes that of seeds appeared. At 50 mM NaCl only specific POX bands of seedlings detected. At higher concentrations of NaCl, the intensity of specific bands of seedlings decreased and some bands appeared likes those of seeds (Fig. 4).

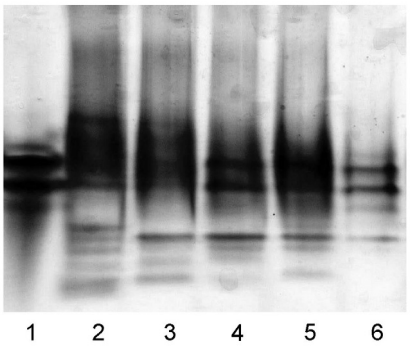


Fig. 4. Activity staining for POX in seeds and seedlings of *T. aphanoneura* under different concentrations of NaCl: seed (1) and seedlings subjected to 0, 50, 100, 150 and 200 mM NaCl (2 to 6).

CAT activity was also measured on PAGE. One band of activity was observed following PAGE analysis of CAT in seedlings (results not shown).

## Discussion

A common feature of *in vitro* cell and callus cultures in response to salt stress is reduction in growth and increase of proline content (*e.g.* Al-Khayri 2002). Similarly, in *Trigonella* species, increasing NaCl stress caused a progressive reduction in growth as expressed in seedlings and calli fresh mass (Tables 1 and 2). Proline, which occurs widely in higher plants, accumulates in larger amounts than other amino acids under salt or water stress. However, according to the contrasting reports on the role of proline in salt tolerance, its use as selection criterion for salt tolerance has been questioned (Ashraf and Harris 2004). Proline content in seedlings and calli of *T. foenum-graecum* decreased at 50 mM NaCl then increased at higher concentrations of salinity. But, proline content in seedlings and calli of *T. aphanoneura* increased under salinity in comparison to that of control. Cherian and Reddy (2003) reported 2- to 9-fold increase in proline content of salinised cells of *Suaeda nudiflora*.

Protein content in seedlings and calli of both species has the similar changes under NaCl stress. Protein content of seedlings and calli increased and decreased under NaCl stress, respectively (Tables 1 and 2). The decrease in protein content of calli at higher concentrations of NaCl may be due to the release of some protein to the media due to osmotic shock (Mass *et al.* 1979) or a decrease in the synthesis of protein (Hall and Flowers 1973). Similarly, Cherian and Reddy (2003) reported decline in protein content in callus culture under NaCl stress. According to the SDS-PAGE analysis of proteins, NaCl induced quantitative and qualitative changes in proteins of seedlings and calli of both species (Figs. 1 and 3). Proteins that accumulate in plants grown under saline conditions may provide a storage form of nitrogen that is re-utilized when stress is over and may play a role in osmotic adjustment. During characterization of salt-induced proteins in tobacco, a 26 kDa protein named as osmotin was detected (Ashraf and Harris 2004).

There are much evidence obtained from various plants showing that the amount and activities of enzymes involved in ROS scavenging, altered by environmental stresses such as drought and salinity. According to Cherian and Reddy (2003) the results obtained with callus tissue were not in total agreement with those observed in whole plant of *S. nudiflora*. The activities of SOD and CAT remained below control level in NaCl treated callus tissues. In our study, NaCl induced alteration in enzyme activity (Tables 1 and 2) and activity staining (Figs. 2 and 4) in two species of *Trigonella* was different from each other.

Moderate salinity stress increased POX activity in seedlings of *T. foenum-graecum*. At higher salinities (150 and 200 mM) POX activity declined to lower than that of control. But POX activity in calli increased steadily to higher than that of control as a function of external salinity. In *T. aphanoneura*, salinity reduced POX activity in seedlings to much lower than that of the control. However, POX activity in calli remained relatively unchanged under salinity except to that of 150 mM NaCl.

Salt stress increased CAT activity in seedings of *T. foenum-graecum* up to 150 mM, but 200 mM NaCl decreased it slightly. CAT activity in seedlings of *T. aphanoneura* increased under salinity with some fluctuation up to 150 mM, but 200 mM NaCl reduced it to lower than that of control. However, there was a pronounced salt induced increase in CAT activity in calli. In accordance to the results obtained by Fornazier *et al.* (2002) in sugar cane, only one band of CAT activity was observed in PAGE analysis of *Trigonella* species.

NaCl did not induced any significant changes in PPO activity in seedlings of *T. foenum-graecum.* In contrast, PPO activity in calli increased steadily with increase in external salinity. PPO activity in seedlings of *T. aphanoneura* increased slightly up to 200 mM NaCl. PPO activity in calli increased slightly at 100 mM NaCl, then decreased to lower than that of control at higher NaCl concentrations (Tables 1 and 2).

## References


Abeles, F.B., Biles, C.L.: Characterization of peroxidase in lignifying peach fruit endocarp. - Plant Physiol. **95**: 269-273, 1991.

Aebi, H.: Catalases. - In: Bergmeyer, H.U. (ed.): Methods of Enzymatic Analysis. Vol. 2. Pp. 673-684. Academic Press, New York 1974.

Agarwal, S., Pandey, V.: Antioxidant enzyme responses to NaCl stress in *Cassia angustifolia*. - Biol. Plant. **48**: 555-560, 2004.

Al-Khayri, J.M.: Growth, proline accumulation, and ion content in sodium chloride-stressed callus of date palm. - *In Vitro* cell. dev. Biol. Plant **38**: 79-82, 2002.

Asharaf, M., Harris, P.J.C.: Potential biochemical indicators of salinity tolerance in plants. - Plant Sci. **166**: 3-16, 2004.

Bartosz, G.: Oxidative stress in plants. - Acta Physiol. Plant. **19**: 47-64, 1997.

Bates, L.S., Waldren, R.P., Teare, I.D.: Rapid determination of free proline for water-stress studies. - Plant Soil **39**: 205-207, 1973.

Cherian, S., Reddy, M.P.: Evaluation on NaCl tolerance in callus cultures of *Suaeda nudiflora* Moq. - Biol. Plant. **46**: 193-198, 2003.

Davis, B.J.: Disc electrophoresis. II. Method and application to human serum proteins. - Ann. New York Acad. Sci. USA **121**: 404-427, 1964.

Fornazier, R.F., Ferreira, R.R., Vitoria, A.P., Nolina, S.M.G.,

Lea, P.J., Azevedo, R.A.: Effect of cadmium on antioxidant enzyme activities in sugar cane. - Biol. Plant. **45**: 91-97, 2002.

Hall, J.L., Flowers, T.J.: The effects of NaCl on protein synthesis in the halophyte *Suaeda maritima*. - Planta **110**: 361-368, 1973.

Holmberg, N., Bulow, L.: Improving stress tolerance in plants by gene transfer. - Trends Plant Sci. **3**: 61-66, 1998.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. - Nature **227**: 680-685, 1970.

Lowry O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J.: Protein measurement with the Folin-phenol reagent. - J. biol. Chem. **193**: 265-275, 1951.

Mass, E.V., Ogata, G., Finkel, M.H.: Salt induced inhibition of phosphate transport and release of membrane proteins from barley roots. - Plant Physiol. **64**: 139-143, 1979.

McCoy, T.J.: Tissue culture evaluation of NaCl tolerance in *Medicago sativa* species: cellular versus whole plant response. - Plant Cell Rep. **6**: 31-34, 1987.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassay with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Niknam, V., Bagherzadeh, M., Ebrahimzadeh, H., Sokhansanj, A.: Effect of NaCl on biomass and content of sugars, proline and proteins in seedlings and leaf explants of *Nicotiana tabacum* grown *in vitro*. - Biol. Plant. **48**: 613-615, 2004.

Olmos, E., Hellin, E.: Mechanisms of salt tolerance in a cell line of *Pisum sativum*: biochemical and physiological aspects. - Plant Sci. **120**: 37-45, 1996.

Raymond, J., Pakariyathan, N., Azanza, J.L.: Purification and some properties of polyphenoloxidases from sunflowers seeds. - Phytochemistry **34**: 927-931, 1993.

Rout, N.P., Shaw, B.P.: Salt tolerance in aquatic macrophytes: possible involvement of the antioxidative enzymes. - Plant Sci. **160**: 415-423, 2001.

Serrano, R., Gaxiola, R.: Microbial models and salt tolerance in plants. - Crit. Rev. Plant Sci. **13**: 121-138, 1994.

Townsend, C.C.: Leguminosae. - In: Townsend, C.C., Guest, E. (ed.): Flora of Iraq. Vol. 3. The University Press, Glasgow 1974.

Van Loon, L.C.: Tobacco polyphenoloxidase. A specific staining method indicating non-identify with peroxidase. - Phytochemistry **10**: 503-507, 1971.

Woodbury, W., Spencer, A.K., Stahmann, M.A.: An improved procedure using ferricyanide for detecting catalase isoenzymes. - Anal. Biochem. **44**: 301-305, 1971.