

IMPACT OF INORGANIC SALT SOLUTIONS ON ANTIOXIDATIVE ENZYMES ACTIVITY AND PHOTOSYNTHETIC PIGMENTS CONTENT IN *TRIGONELLA FOENUM-GRÆCUM* SEEDLINGS

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Abstract: Fenugreek (*Trigonella foenum-graecum*) is known to have several pharmacological effects such as hypoglycemia, hypocholesterolemia, gastroprotective, chemopreventive, antioxidant, antiinflammatory, antipyretic and appetite stimulation attributes. This plant is known to contain many secondary metabolites like alkaloids, flavonoids, salicylate, and nicotinic acid. Present investigation was undertaken to study the effect of salinity on fenugreek antioxidative defense mechanism. The response of the superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) activities as well as the pigment content in fenugreek seedlings was investigated after 24 days of treatment with inorganic salt solutions. It was used for treatment different concentration of NaCl alone and in combination with CaCl₂ as well as MgCl₂. The fenugreek treatment was effected to elucidate the adverse effect of NaCl salinity and its possible amelioration by CaCl₂ or MgCl₂. Generally, all treatments diminished the activity of antioxidant enzymes take in the study with one exception (150mM NaCl+10mM CaCl₂ in case of SOD). Only a few concentrations (50mM NaCl, 100mM NaCl, 50mM NaCl+10mM CaCl₂, 150mM NaCl+10mM CaCl₂, 100mM NaCl+20 MgCl₂) increased the fenugreek soluble protein content. Both CaCl₂ and MgCl₂ treatments applied alone and in combination determined an increase of photosynthetic pigments (Chl *a*, Chl *b* and carotenoids) contents compared with control

INTRODUCTION

Soil salinity is a major danger to crop yield and salt stress causes huge losses of agriculture productivity worldwide. Thus, salt stress presents an increasing threat to plant agriculture. On the other hand, salinity like other stresses can affect the growth of plant by reduces the quality and productivity but in further stages can cause plant death (Giri et al., 2003, Al Karaki, 2006, Dajic, 2006, Rahdari and Seyed, 2011). The most harmful effects of salinity on plants include ion toxicity, water deficit (Liu and Van Standen, 2001) and nutrient imbalance (Grattan and Grieve, 1999). Excessive sodium (Na⁺) inhibits growth of many salt sensitive plants and glycophytes, which includes most crop plants. It is known that calcium increase salinity tolerance and mitigates the adverse effects of saline conditions on plant growth (Jaleel et al., 2007).

Some researches that reported that by adding of Ca²⁺ and K⁺ in nutrition solution it is reduced the degree of stress and protect them from NaCl stress (Cramer, 2002). Thus, supplementing the medium with Ca²⁺ alleviates growth inhibition by salt of glycophytes and being possible involved in signal transduction involving new gene expression (Trofimova et al., 1999) under oxidative stress. In addition, in plant cells, calcium functions as a second messenger, coupling a wide range of extracellular stimuli to intracellular responses (Sneeden and Formm, 2001), and plays important role in plant growth and development (Arshi et al., 2006). Ca²⁺ is also a primary second messenger in signal transduction and regulates physiological and biochemical processes in the responses of plants to extracellular adverse abiotic environments (Bowler and Fluhr, 2000). Regarding the other major cation Mg²⁺, is cofactors required for the activity of different enzymes, including enzymes involved in respiration and photosynthesis and in addition, it is implicated in the ring structure of the chlorophyll molecule. Concerning the specific roles on the mechanisms of response of plants to high soil salinity and salt tolerance there are very little known. However, there are some experimental data indirectly suggesting that Mg²⁺ could play a similar role to that of Ca²⁺, protecting plant cells from the deleterious effects of NaCl (Grigore et al. 2012).

On the other hand, all variable environmental stresses (salt stress, drought, cold, heat salt stress, toxic heavy metals etc) have been reported to lead in the cell at the excessive generation of harmful reactive oxygen species (ROS) such as superoxide anion (O₂⁻), hydroxyl radical (OH⁻) and hydrogen peroxide (H₂O₂) (Mittler, 2002). Major production areas of these toxic radicals in plant cells are located in the electron transfer chain of mitochondria and chloroplast and peroxisome.

Plants have developed some protection mechanisms against the harmful effects of ROS and to alleviate their deleterious effects. To keep the levels of active oxygen species under control, plants generates non-enzymatic and enzymatic antioxidant systems to protect cells against the harmful effects of ROS (Mittler, 2002). Antioxidant enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and peroxidase (POD, EC 1.11.1.7) are among the major antioxidant defence systems of plants (Xu et al., 2008). The major antioxidant defense system contains also the non-enzymatic antioxidants (β-carotenes, ascorbic acid, α-tocopherol, reduced glutathione). Measurements of the

antioxidant enzyme activity may provide information concerning the degree of exposure of plant tissues to reactive oxygen species and the link between salt stress and ROS.

Fenugreek (*Trigonella foenum-graecum* L.) is an annual dicotyledonous plant belonging to the family *Fabaceae* mainly used as medicinal plant. It is commonly used as condiment food preparation for its nutritive and restorative properties and has been used in folk medicine for centuries including diabetes (Eidi et al., 2007). The whole plant is used as forage and vegetable, with the seed (whole, powdered, in flour or roasted). It is considered a good soil renovator and often grown as break crop for cereal. Fenugreek is fairly tolerant to salt and can adapt to variable climatic conditions and growing environmental (Duke 1986 and Petropoulos 2002). It has a broad spectrum of therapeutic properties and also studied as animal growth promoter (Atefeh Sheikhlar, 2013). Seeds from *T. foenum graecum* are a good source of protein (20-30%) high in lysine and triptophan, fat (6.53%), ash content (3.26%), crude fibre (6.28%), energy (394.46% Kcal/100g of seed) (Khan et al., 2011). It is a good source of vitamin A and D as well as some secondary metabolites such as alkaloids (trigonelline, gentianine, carpaine) glycosides (steroidal saponin) polyphenolic compounds (coumarin, scopoletin, chlorogenic acid, caffeic acid) (Valette et al., 1984, Patil and Jain, 2014). The fenugreek contents of protein and steroidal saponins are comparable to those of soybean (Sheikhlar, 2013).

In order to elucidate the adverse effect of NaCl salinity and its possible amelioration by adding CaCl₂ or MgCl₂ solutions (in combination with NaCl or alone), in the present study antioxidative enzymes (SOD, CAT, POD) activity, protein content and pigment amount in fenugreek seedlings were investigated after 24 days of treatment with inorganic salt solutions.

MATERIAL AND METHODS

Plant material, treatment and growth conditions

In order to determine the effect of inorganic salt solutions on some indices in *Trigonella foenum-graecum* L. seeds an experiment was conducted in laboratory conditions based on completely randomized design with three replications. Fenugreek (*Trigonella foenum-graecum*) seeds were obtained from Agricultural Research and Development Station, Secuieni Neamt. Intact seeds, which were homogeneous and identical in size and colour, and free from wrinkles, were chosen. These seeds were then sterilized with sodium hypochlorite 10% for 30 seconds and were washed with sterile distilled water. After that, 30 fenugreek seeds were grown in pots salinized with sodium chloride solution (50mM NaCl, 100mM NaCl, 150mM NaCl, 50mM NaCl together with 10mM CaCl₂, 100mM NaCl together with 10mM CaCl₂, 150mM NaCl together with 10mM CaCl₂, 50mM NaCl together with 20mM MgCl₂, 100mM NaCl together with 20 MgCl₂, 150mM NaCl together with 20mM MgCl₂, 10mM CaCl₂, 20mM MgCl₂) and distilled water (control). Solution of sodium chloride and distilled water were applied as drench (10 mL per pot) after every 3 days. All physiological and biochemical analyses were performed at 24 days old when seedlings were uprooted randomly.

Enzymes activity assay

Preparation of enzyme extracts

Fenugreek seedling sample (0.3g) were homogenized with phosphate buffer (pH=7.5). After that the homogenates were centrifuged at 15,000×g for 15 min. at 4 °C and the supernatants were used for enzyme assays.

Superoxide dismutase (SOD) activity was estimated by recording the decrease in absorbance of superoxide-nitroblue tetrazolium complex by the enzyme (Atenie et al., 2008). About 3 mL of reaction mixture, containing 0.1 mL of 1.5 mM nitroblue tetrazolium (NBT), 0.2 mL of 0.1 M EDTA, 2.55 mL of 0.067 M potassium phosphate buffer, and 0.01 mL of enzyme extraction, were taken in test tubes in duplicate from each enzyme sample. One tube without enzyme extract was taken as control. The reaction was started by adding 0.05 mL of 0.12 mM riboflavin and placing the tubes below a light source of 215 W florescent lamps for 5 min. The reaction was stopped by switching off the light and covering the tubes with black cloth. Tubes without enzyme developed maximal colour. A non-irradiated complete reaction mixture, which did not develop colour, served as blank. Absorbance was recorded at 560 nm and 1 unit of enzyme activity was taken as the quantity of enzyme that reduced the absorbance reading of samples to 50% in comparison with tubes lacking enzymes.

Peroxidase (POD) activity was determined spectrophotometrically by measuring the oxidation of o-dianisidine at 540 nm (Moller and Ottolenghi, 1966) with slight modification. The reaction was started by adding 0.1 H₂O₂ 0.05% on mixture reaction containing 0.2 mL of enzyme extraction, 0.8 mL distilled water and 1.5 mL 1% o-dianisidine. After 5 min. the reaction was stopped with 2.5 mL H₂SO₄ 50%. One unit of POD activity was expressed as the amount of enzyme that produced a change of 1.0 absorbance per min.

Catalase (CAT) activity was measured according to the method described by Sinha, 1972. Briefly, the assay mixture consisted of 0.4 mL phosphate buffer (0.01 M, pH 7.0), 0.5 mL hydrogen peroxide (0.16 M) and 0.1 mL enzymatic extract in a final volume of 3.0 mL. About 2 mL dichromate acetic acid reagent was added in 1 mL of reaction mixture, boiled for 10 min, cooled. Changes in absorbance were recorded at 570 nm. CAT activity was expressed as the

amount of enzyme needed to reduce 1 μmol of H_2O_2 per min. The activity of these enzymes (SOD, POD and CAT) was expressed as unit per mg proteins (U/mg protein).

Photosynthetic pigment content assay

Chlorophyll-a, chlorophyll-b and carotenoids content assays were performed according to Lichtenthaler 1987. Thus fresh leaves of samples were homogenized in 80% acetone and then were centrifuged at 4°C for 15 min (3000 rpm). Finally the volume was made to 5 mL and used then for the analysis. Absorbances were determined at 645, 663 and 470 nm respectively and pigment contents were evaluated and expressed in mg/g fresh weight (FW) using the following equations:

Chlorophyll *a* (Chl *a*) = $(11.24 \times A_{662} - 2.04 \times A_{645})$

Chlorophyll *b* (Chl *b*) = $(20.13 \times A_{645} - 4.19 \times A_{662})$

Carotenoids = $[(1000 \times A_{470} - 1.9 \times \text{Chl } a - 63.14 \times \text{Chl } b) / 214]$

Protein Estimation

Total soluble protein content was measured according to Bradford (1976) using bovine serum albumin (BSA) as a protein standard. Fenugreek seedling sample (0.3g) were homogenized with 0.1 M phosphate buffer (pH=7.5) and then at 4°C. Supernatants was pipetted in spectrophotometer cuvettes and absorbance was measured using a UV-vis spectrophotometer at 595 nm

Statistical analysis. All experiments were carried out with three independent repetitions and the results were expressed as the mean values \pm standard deviation (ES).

RESULT AND DISCUSSIONS

Effect of salinity on photosynthetic pigment content

Salinity did not affect the photosynthetic pigment of *T. foenum-graecum*: in terms of declining their amount as seen in Tab. 1. Thus, the content of chlorophyll *a*, chlorophyll *b* and carotenoids varied only slightly among treatments. Applying of inorganic salts treatment on *T. foenum-graecum* increase the amount of pigment compared to control.

The general trend for effect of inorganic salt treatment on *T. foenum-graecum* seedling reflect an enrichment in the pigment amount which could be due to an increase in the number of chloroplasts in stressed seedlings. The higher content was observed for all photosynthetic pigment studied at treatment only with 10mM CaCl_2 (0.315 mg/g FW, 0.126 mg/g FW and 0.024 mg/g FW for Chl *a*, Chl *b* and carotenoids, respectively).

As a matter of fact, there has been knowledge on increase of chlorophyll content in saline environment depending on salt levels (Romero-Aranda et al., 2001). Our data regarding an increase in both chlorophyll *a* and chlorophyll *b* with the increase of salinity agree with results reported by Misra et al., 1997. They observed that stressing rice seedling *Oryza sativa* L. with sodium chloride increased significantly the chlorophyll content of seedlings, which were 15 days old. Also, another study it was mentioned by Jamil et al., 2007 which reveal that increased concentrations of sodium chloride (50 and 150mM) enhance significant the total chlorophyll content of sugar cane leaves (*Beta vulgaris* L.). Increasing of leaf chlorophyll *a* content, chlorophyll *a/b* and chlorophyll *a + b* and carotenoids under 30mM level of salinity stress was also reported by Pinheiro et al, 2008 on *Ricinus communis* at both 38 days after germination.

Aurangzeb et al., 2013 find that the chlorophyll contents were increased in all the wheat cultivars with the addition of salt at tillering stage. Sensitive cultivars had higher and lower chlorophyll contents compared to tolerant cultivars at first booting and flowering stages, respectively. It was observed that chlorophyll content in Sensitive cultivars was not increased so much at last stage. Ghogdi et al., 2012 has observed at wheat the almost same results. Another study performed by Rahdari et al., 2012 reported also, an increase of both chlorophyll levels in Purslane leaves and the highest level of chlorophyll *a* and *b* by order in 150 and 200 Mmol of NaCl was observed. Higbie et al., 2010 remarked a significantly increase of chlorophyll content index (CCI) in 3 of the 6 genotypes of cotton at 21 days after treatment with NaCl.

Our results are in accordance with the study of Jaleel and Azooz, 2009, where the applied treatment comprising sodium chloride and calcium chloride (100mM NaCl, 5 mM CaCl₂ and 100mM NaCl +5mM CaCl₂) altered the prolin metabolism of *Withania somnifera* plants. Combined application of NaCl and CaCl₂ led to a decrease of chlorophyll a, chlorophyll b, total chlorophyll and carotenoids contents, a decrease activity of proline oxidase and an increase activity of γ -glutamyl kinase activity. Situation was inverse when it was compared the parameter levels with the plants treated with NaCl only. Thus, application of CaCl₂ to NaCl-treated plants resulted in an increase in content of chlorophyll a, chlorophyll b, total chlorophyll along with an increase in PRO oxidase activity, and a decrease in γ -glutamyl kinase activity.

Table 1. Changes in chlorophyll-a (Chl-a), chlorophyll-b (Chl-b), and carotenoids (Car) (mg.g⁻¹ FW) concentrations in 24 days old fenugreek seedling after of treatment induced by inorganic salt solutions

	Chlorophyll a (mg/g FW)	Chlorophyll b (mg/g FW)	Carotenoids (mg/g FW)
Control	0.138	0.045	0.009
50mM NaCl	0.218	0.079	0.01
50mM NaCl+10mM CaCl ₂	0.225	0.092	0.023
50mM NaCl+20mM MgCl ₂	0.23	0.097	0.017
100mM NaCl	0.232	0.091	0.016
100mM NaCl+10mM CaCl ₂	0.289	0.118	0.021
100mM NaCl+20 mM MgCl ₂	0.268	0.104	0.013
150mM NaCl	0.226	0.093	0.011
150mM NaCl+10mM CaCl ₂	0.213	0.096	0.012
150mM NaCl+20mM MgCl ₂	0.311	0.13	0.015
10mM CaCl ₂	0.315	0.126	0.024
20mM MgCl ₂	0.246	0.101	0.017

Effect of salinity on SOD activity

SOD activities in 24 days old fenugreek seedlings under the effect of inorganic salt stress and control are present in Figure 1. The results show that comparing with control the enzymes SOD activity was decreased after treatment in all seedling variants except that which was grow on 150mM NaCl +10mM CaCl₂ concentration. SOD is thought to be one of the most important defense systems, which detoxifies superoxide anion free radicals, by the formation of H₂O₂. Subsequently, H₂O₂ is scavenged into H₂O and O₂ by CAT and a variety of peroxidase compounds such as APX and glutathione peroxidase.

If at concentration 50 mM NaCl the application of combined treatment with CaCl₂ or MgCl₂ has not alleviated noticeable the toxicity of sodium chloride on SOD activity, the effect was opposite at the other concentrations. Thus, at both concentrations, 100mM and 150 mM NaCl, application of MgCl₂ reduced the SOD activity with 21% and 25%, respectively. In addition, the strong decrease in SOD activity (55.22%) was induced by 20mM MgCl₂ concentration when was alone applied. On the other hand, application of CaCl₂ in combination

with 100mM NaCl diminished (23%) the activity of this enzyme while CaCl₂ in combination with 150mM NaCl increase the activity also with 23%.

Decrease of SOD activity caused by stress may be regarded as an omen of plant toxicosis. Our results are in accordance with those of other authors. Thus, Rahnama, Ebrahimzadeh, 2006 reported that in *Solanum tuberosum* cultivars SOD activity decreased when callus were grown in the presence of NaCl. Moussa, 2006, finds that chloride stress decreased the SOD and CAT activity in maize seedling. The author suggested that silicon partially offset the negative impacts and increased tolerance of maize to salinity by enhancing SOD and CAT activities.

On the other hands, Khan et al., 2012 showed that application of CaCl₂ alleviated NaCl stress on mustard plants by enhancing the activities of SOD, CAT, POD and glutathione reductase as well as by enhancing proline and glycinebetaine accumulation which is manifested in the tolerance of plants to salinity stress.

According to Jaleed et al., 2007c, supplementing the growth medium with Ca alleviated the salt-inhibited plant growth in glicophytic plants. On NaCl-stressed plants of *Vigna radiate*, Manivannan et al., 2007 assessed the ameliorating effect of CaCl₂. The contents of proline and glycinbetaine as well as the activities of SOD, APX and CAT enzymes were increased by treatment of NaCl or CaCl₂ applied alone. However, application combined of CaCl₂ with NaCl altered the overall plant metabolism, ameliorating the deleterious effects of NaCl stress and increasing the vegetative growth of plants.

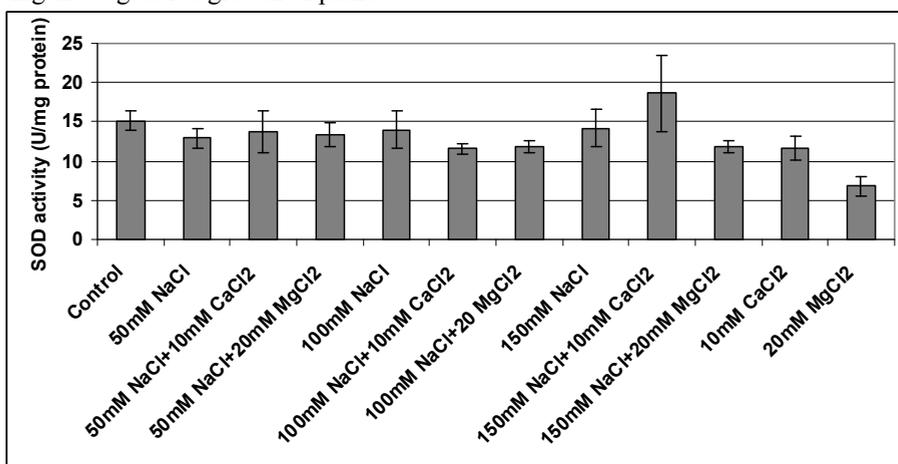


Fig. 1. Change in superoxid dismutase activity in 24 days old fenugreek seedling after treatment induced by inorganic salt solutions (means ± ES, n = 3).

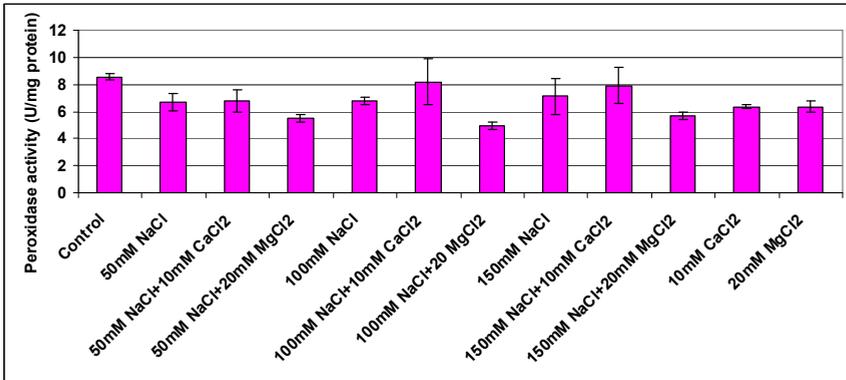


Fig. 2. Change in peroxidase activity in 24 days old fenugreek seedling after treatment induced by inorganic salt solutions (means \pm ES, n = 3).

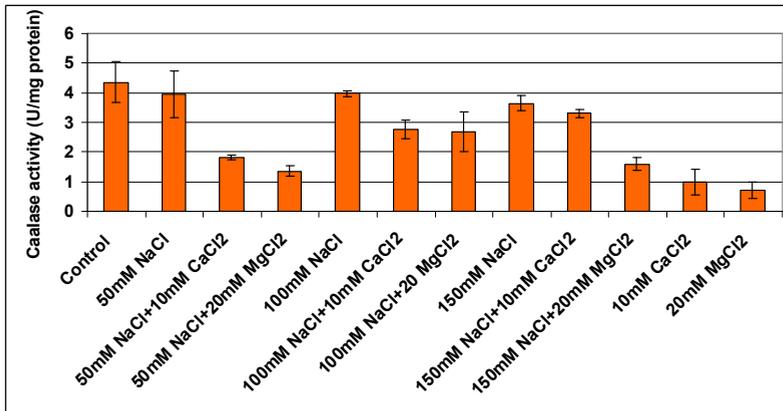


Fig. 3. Change in catalase activity in 24 days old fenugreek seedling after treatment induced by inorganic salt solutions (means \pm ES, n = 3).

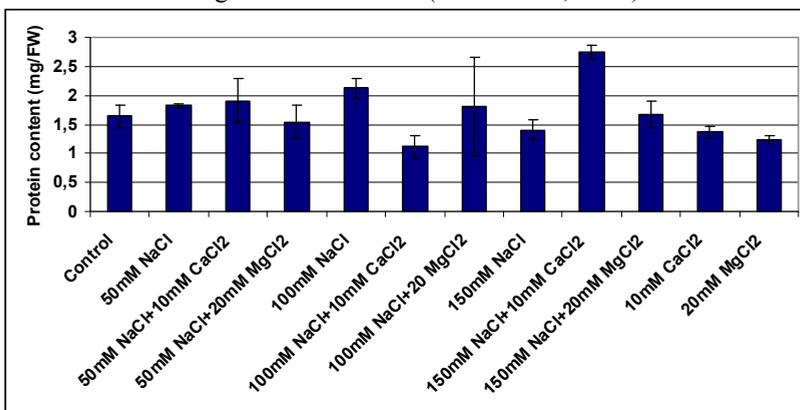


Fig. 4. Change in soluble protein content in 24 days old fenugreek seedling after treatment induced by inorganic salt solutions (means \pm ES, n = 3)

Effect of salinity on POD activity

After 24 days, the peroxidase activity in fenugreek seedlings evidenced variations between treatments with concentration of NaCl applied alone or in combination with CaCl₂ or MgCl₂ (Figure 2). The results indicate that the treatment with NaCl in combination with MgCl₂ determined the most diminution of POD activity in fenugreek seedling. Thus, the highest decrease, 42% was in case of combination of 100 mM NaCl with MgCl₂. Knowing that the application of CaCl₂ mitigated the adverse effects caused by NaCl stress, Goharrizi et al., 2011, investigated the effect of different salinity levels both on unstressed and salt-stressed walnut plant, using CaCl₂ as stress amelioration source. They found that the POD and CAT activities increased in parallel with the increase in salt levels in the leaves of tolerant genotypes of walnut treated with CaCl₂. However, in all the treatment, POD and CAT activities were reduced significantly from 6 to 10 days after beginning the salt stress.

Applied treatment of MgCl₂ and CaCl₂ alone evidenced the same effect on POD activity, the diminished being by 25% in comparison with control. Application of combined of NaCl and CaCl₂ revealed an increase on POD activity in case of 100mM and 150mM NaCl concentrations if compared with plants stress with NaCl corresponding concentrations.

Effect of salinity on CAT activity

As a result to the exposure to different concentrations NaCl, the treatment application of CaCl₂ and MgCl₂, reduced the CAT activity in fenugreek seedling comparing to control. The decrease of this enzyme activity was higher by combined application of sodium chloride with MgCl₂ (68% with 50mM, 38% with 100mM and 68% with 150mM) than CaCl₂. On the other hand, a drastic decrease on CAT activity, with reference to control, was caused by alone application of CaCl₂ and MgCl₂, by 77% and 83%, respectively. The CAT inactivation under saline conditions is attributed either to a possible modification of its biosynthesis (Feierabend and Dehne, 1996). In agree with other results, reduction in catalase activity under salt stress may result in H₂O₂ accumulation and may be associated with its tolerant mechanism through signal transduction (Jaleel et al. 2007c).

Effect of salinity on soluble protein content

Biochemical response regarding soluble protein in fenugreek seedling after 24 days old upon the treatment with inorganic salt is extremely different (Figure 4). Between treatments with sodium chloride applied alone, only concentration 150 mM NaCl leads to a decrease of soluble protein content comparative with control. On the contrary, the combined treatment with CaCl₂ and MgCl₂ evidenced a decrease of protein content in case of 100mM NaCl concentration. The increased of soluble protein content could reflect a defence response to the cellular damage provoked by salt concentrations in soil. Applied of CaCl₂ and MgCl₂ alone decrease by 16% and 24%, respectively, the level of protein comparatively with control.

CONCLUSIONS

In conclusion, the results of this study regarding the effect of treatment with eleven solutions of salts (NaCl, CaCl₂ and MgCl₂) applied alone or combined at *Trigonella foenum graecum* seeds show variations of the parameters investigated.

Application of combined treatment with CaCl₂ or MgCl₂ at concentration 50 mM NaCl has not alleviated noticeable the toxicity of sodium chloride on SOD activity. The MgCl₂

treatment in combination with NaCl reduced the SOD activity with 21% and 25%, respectively, at 100mM and 150 mM NaCl.

Between the combined treatment with MgCl₂ and CaCl₂, it seems that those with MgCl₂ caused a more pronounced reduction of peroxidase and catalase activities, both in relation to the control and singular NaCl treatments corresponding.

Generally, both treatments applied alone and in combination determined an increase of photosynthetic pigments (Chl a, Chl b and carotenoids) contents compared with control. In addition, the combined treatment with CaCl₂ and MgCl₂ led to enhance of pigment content comparatively with singular NaCl treatment corresponding. The highest content of all photosynthetic pigments was remarked by applied alone of CaCl₂.

Combined treatments of NaCl and CaCl₂ caused an increase in protein content by 16% and 67%, at 50 mM and 150 mM respectively, comparative to control. Meanwhile, the combined MgCl₂ treatments caused a milder increase by 11% and 2% compared to control at 100 mM and 150 mM NaCl.

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