

EFFECT OF EXOGENOUS PROLINE ON DURUM WHEAT (*Triticum durum* Desf.) UNDER NaCl STRESS

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Abstract. This experiment was conducted to determine the effect of exogenous proline (20 mM) on the physiological and biochemical behavior of durum wheat seedlings subjected to a salt constraint (10 g/l NaCl). This effect was evaluated through the estimation of plant water status (leaf relative water content), photosynthetic pigments, sugars content, water soluble proteins content, the osmotic adjustment (endogenous proline and glycine betaine), the lipid peroxidation (the membrane integrity and the malondialdehyde content) and the activity of the antioxidant enzyme catalase in wheat plantlets. Results obtained show that the saline stress affects the majority of the studied parameters. However, exogenous application of proline seems to attenuate the negative effects of the saline stress by the improvement of the content of total chlorophyll, proteins, glycine betaine and the relative electrolyte leakage. These results suggest the capacity of the exogenous application of proline to improve the tolerance of the salt-stressed wheat plants.

Keywords: exogenous proline, salt stress, *Triticum durum*, osmolyte, antioxidant.

INTRODUCTION

Salinity stress remains one of the most serious environmental problems which substantially hampers crop productivity in arid and semi-arid areas [29]. The problem of soil salinity is further aggravated through the use of poor quality water for irrigation and inadequate drainage [35]. In Algeria, for instance, salt affected soils occupy an estimated area of 3 million hectares, or approximately 40% of the total area of Algerian's arable land [26]. While wheat and barley are the main cereal crops, durum wheat is by far the most cultivated cereal in Algeria, however, wheat crop often confront abiotic stresses such as salinity [26]. Salt stress affects plant physiology at whole plant as well as cellular levels, it affects physiological and biochemical processes which result in reduced biomass production [50]. Adverse effects of high concentrations of salts cause ion imbalance and hyperosmotic stress in plants. As a consequence of these primary effects, secondary stresses such as oxidative damage often occur [73]. Under these unfavourable environmental conditions, plants initiate some defensive and protective mechanisms to allow them to cope with stress for survival and growth; one of them is associated with changes in metabolites including the accumulation of osmolytes such as proline [27, 28, 67]. Proline appears to be the most widely distributed osmolyte accumulated under stress conditions in plants. Although proline can be synthesized from either glutamate or ornithine, glutamate is the primary precursor in osmotically stressed cells. The biosynthetic pathway involves two important enzymes *pyrroline 5 carboxylate synthase* and *pyrroline carboxylate reductase* [59, 64, 68]. Most stress studies have demonstrated the existence of a strong correlation between the increased cellular proline levels and plant tolerance to salt stress [22, 54], its accumulation under stress has been assigned several roles [61]. Proline acts

as an osmo-protectant, and plays an important role in osmotic balancing, protection of sub-cellular structures, enzymes and in increasing cellular osmolarity (turgor pressure) that provide the turgor necessary for cell expansion under stress conditions [54, 43]. Proline also plays roles in buffering cellular redox potential and scavenging free radicals under NaCl stress [68]. Other roles have been proposed for proline accumulation: a sink of energy, a nitrogen-storage compound [60] and a signaling molecule [68, 39]. As a compatible solute, the amino acid proline has shown a ray of hope in combating such stresses in crop plants through its overexpression in the appropriate cells via genetic engineering. Exogenous application of proline has also gained tremendous impetus in developing stress tolerance in various plant species [65, 58]. This alternative strategy can provide osmoprotection and improve the production of crops growing under saline conditions [48, 31]. However, the mechanisms of exogenous proline under stress conditions are yet to be fully understood [33] and according to our knowledge, insufficient information are available about the impact of proline on salt-stressed durum wheat. Thus, the present study was initiated to examine the effect of exogenous application of proline on durum wheat at the physiological and biochemical level during the early growth stages under NaCl stress.

MATERIAL AND METHODS

Plant material

Taking into consideration its great productive potential since its introduction in 1989 by the technical institute of ITGC of Setif (Algeria) and its high nutritional value, the cultivar of durum wheat *Waha* was used in this study. It is widespread mostly in Algerian semi-arid areas. The seeds were obtained from the National Center of Control and Certification of seeds and plants El-Harrach, Algeria. After

disinfection with sodium hypochlorite 6 % for 20 minutes and an extensive rinse with sterile distilled water (five times), seeds were germinated in wet Whatman paper at 25°C. Seven days after germination, healthy seedlings of similar size were transplanted to containers (11x22cm) of hydroponic system containing at first time the distilled water for their acclimatization. Each container had twenty plantlets. After three days pre-culture, the distilled water is replaced with the nutrient solution [19] at a volume of 200 ml per container and the seedlings were then cultured in four different nutrient solutions designed as : *Control*: plants receiving the standard nutrient solution; *NaCl treatment*: plants receiving the nutrient solution supplemented with 10 g/l NaCl (171.11 mM); *Proline treatment*: plants receiving the nutrient solution and 20 mM supplementary L-proline; *NaCl + Proline*: plants receiving the nutrient solution supplemented with 10 g/l NaCl and 20 mM supplementary L-proline. The selection of the concentration of proline (20 mM) was based on preliminary tests. The nutrient solution was continually aerated using an air pump and was renewed every two days. The experiment was carried out in conditioned growth chamber (16h light/8h dark photoperiod, 25°C). Twenty one days after treatment corresponding to the third leaf stage, plant materials were carefully sampled and separated into roots and shoots.

Estimation of the leaf water content and chlorophyll content

Relative water content (RWC) was determined according to Clarke and McCaig [18]. Total chlorophyll was estimated in 80% acetone-extracted leaves according to the method described by Lichtenthaler [44].

Estimation of the total soluble sugars and water soluble proteins contents

Total soluble sugars of leaf tissues were extracted with 80% ethanol by heating at 90°C and their content were estimated by using anthrone reagent as described by McCready et al. [46]. Optical density of the sample extract was determined spectrophotometrically at 630 nm. The total soluble proteins content was quantified at 595 nm by the classical method using Biorad reagent as described by Bradford [12] with bovine serum albumin as a standard.

Estimation of the proline and glycine betaine content

Quantitative determination of proline was performed by following the colorimetric method of Magné and Larher [45]. Briefly, 50 mg of leaf samples were homogenized in 0,5 ml of distilled water and proline content of extracts was estimated using acid-ninhydrine as color reagent and L-proline as standard. Absorbance of the resulting organic layer was spectrophotometrically measured at 520 nm. Leaf glycine betaine was extracted from dried and powdered material with distilled water at 100°C for one hour.

After filtration, the extracts were diluted 1:1 with 2N HCl solution and the glycine betaine content was determined spectrophotometrically at 365 nm on the organic layer after reaction with potassium tri-iodide solution (KI-I₂) according to the of Grieve and Grattan method [30].

Estimation of the membrane integrity: lipid peroxidation and electrolytes leakage

The lipid peroxidation was measured by estimating the level of malondialdehyde as indicator of membrane stability. MDA was assayed by thiobarbituric acid reactive substances (TBARS) contents and the concentration of MDA was calculated from the absorbance at 532 nm and measurements were corrected by subtracting the absorbance at 600 nm for unspecific turbidity using the MDA extinction coefficient of 155 mM⁻¹cm⁻¹ according to [32]. Total electrolyte leakage was measured using an electrical conductivity meter on excised sections of young leaves of each treatment as described by [23].

Assay of the catalase activity

Catalase is one of antioxidant enzymes, necessary for breaking down the accumulated H₂O₂ under oxidative stress conditions. Fresh leaf tissue (300 mg) was used for enzyme assay and samples were homogenized with extracting buffer. An aliquote was used to determine the protein content of the extract according to Bradford (1976) using bovine serum albumin as the standard. Catalase activity was assayed in a reaction solution as described by Dorey et al. [24]. The reaction was started by the addition of H₂O₂ and the activity was estimated by the decrease in absorbance at 240nm for 1 min as a consequence of H₂O₂ consumed using extinction coefficient of 36 M⁻¹cm⁻¹.

Statistical analysis

The experiment was set up in randomized complete design. Experimental data were subjected to one-way analysis of variance (ANOVA). Differences were reported as statistically significant at P < 0.05 and the significant differences were highlighted using the Newman-Keuls test. The statistical analyses was performed using SPSS 10.0 software and Microsoft ® Excel 2007 Analysis. The results are shown in graphs and given as the mean±SE (mean: value of three replicates; SE: standard error of mean).

RESULTS

Leaf relative water content (LRWC) and chlorophyll content

As shown in figure 1a, the water content decreased in response to salt stress which is about of 30.08 % in wheat-stressed plantlets in comparison with control treatment. However, stressed plants fed with proline had slightly higher water contents compared to those stressed with NaCl alone, but this increase is not

significant. Figure 1b shows that chlorophyll content decreased under saline conditions (44.5 % in comparison to the control). Significant increase (up to a 2.5-fold) in chlorophyll content was measured in proline-treated plantlets under salt-stress conditions compared to the NaCl treatment without exogenous proline.

Total soluble sugars and water soluble proteins contents

Wheat plantlets grown within NaCl increased their soluble sugars content compared with control plants (Fig. 2a). In the other hand, there was no significant difference in the plant sugar content between proline treated plants under salinity stress and salt-stressed plants. Under unstressed conditions, exogenous proline kept it comparable to that of the control. Results analysis show that, the salt stress has substantially decreased the water soluble proteins content of leaves compared to the control (Fig. 2b). In response to proline supplied externally, the proteins content of plantlets was significantly increased.

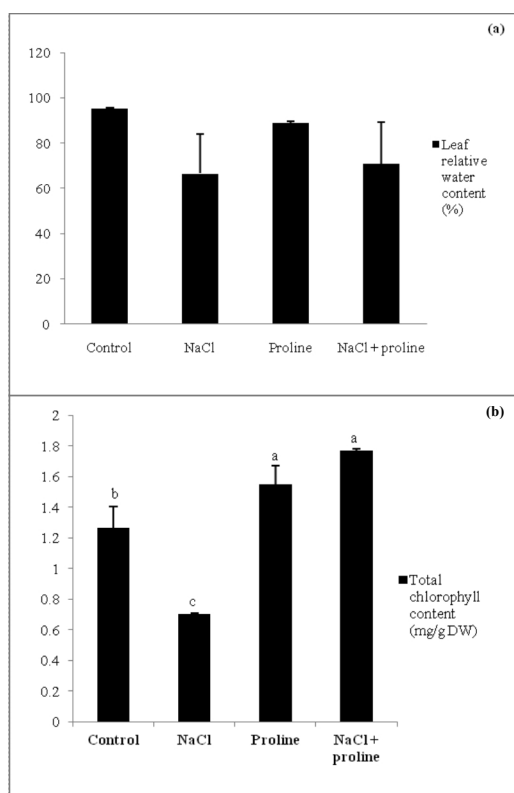


Figure 1. Effect of 20 mM exogenous proline application on leaf relative water content (a) and total chlorophyll content (b) of durum wheat plantlets exposed to 10g/l NaCl. Different letters represent a significant difference ($P < 0.05$) between treatments. DW: dry weight.

Proline and glycine betaine content

Our results shows that the internal proline content (Fig. 3a) increased slightly under salinity stress than that of control. The higher level of the endogenous proline was observed in response to exogenous proline in stressed plantlets compared to those stressed with NaCl alone, which means that its level can be resulted additionally by the endogenous and exogenous proline, although statistical analysis did not show that this difference was significant. The glycine betaine content of wheat increased in treatment with NaCl and it was higher than that of the control (about 72% of increase). Salt-stressed plants fed with 20mM proline had significantly higher glycine betaine content compared to plants stressed with NaCl alone (Fig. 3b).

Membrane integrity: the malondialdehyde content and electrolytes leakage

As shown in figure 4a, the malondialdehyde content tends to show greater accumulation under salt stress. The proline treatment decreased the MDA content under stressed conditions (by 35.74 %). However, there was no significant difference between stressed plants and plants grown with NaCl plus proline. In the other hand, the amount of total electrolyte leakage significantly decreased in plants grown in NaCl and proline conditions (by 61.91%) (Fig. 4b) indicating a more stability of cellular membranes against salinity stress in response to exogenous proline application.

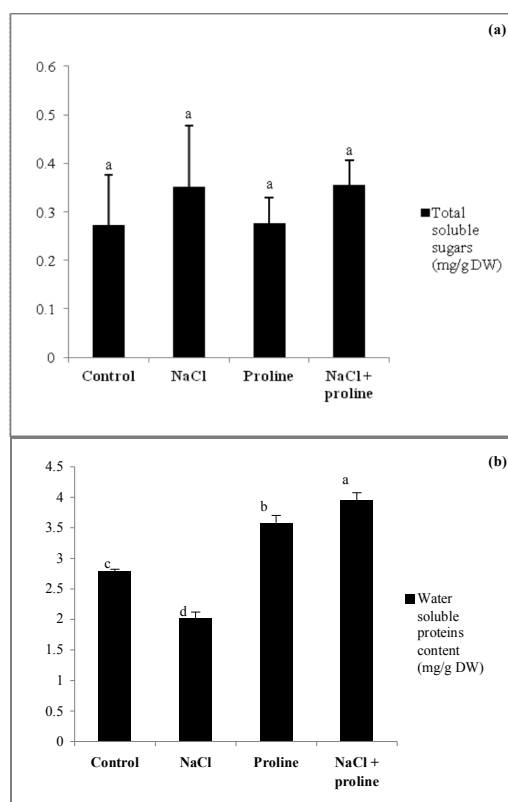


Figure 2. Effect of 20 mM exogenous proline application on shoot soluble sugars (a) water soluble protein contents (b) of durum wheat plantlets exposed to 10g/l NaCl. Different letters represent a significant difference ($P < 0.05$) between treatments.

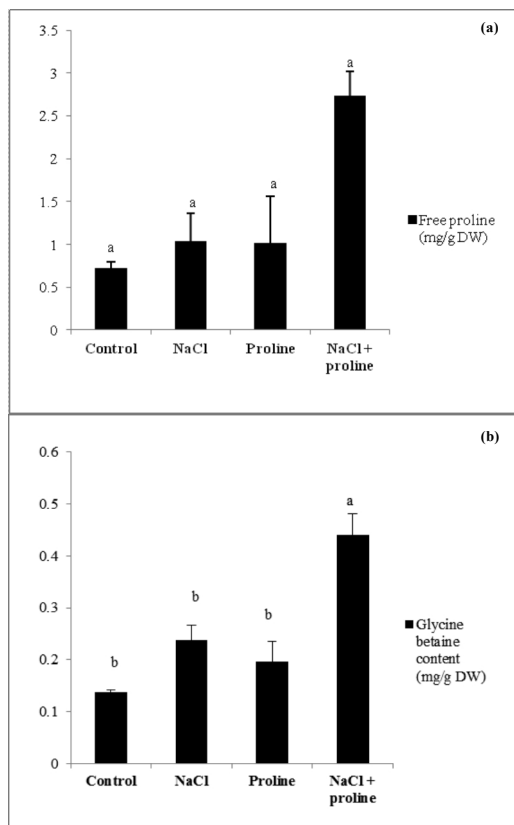


Figure 3. Effect of 20 mM exogenous proline application on free proline (a) and on glycine betaine contents (b) of durum wheat plantlets exposed to 10g/l NaCl. Different letters represent a significant difference ($P<0.05$) between treatments.

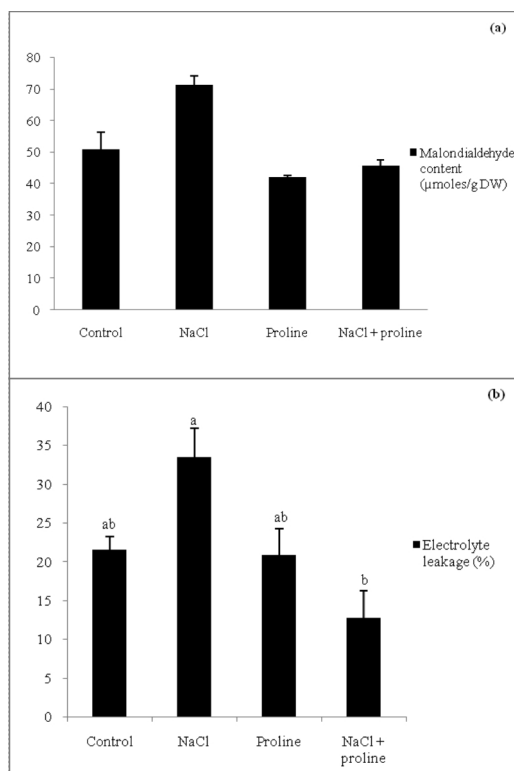


Figure 4. Effect of 20 mM exogenous proline application on malondialdehyde content (a) and on electrolyte leakage (b) of durum wheat plantlets exposed to 10g/l NaCl. Different letters represent a significant difference ($P<0.05$) between treatments.

Catalase activity

Under stressful conditions, the catalase activity decreased in relative to the control. In the presence of proline, this enzyme activity was higher than in its absence (by 40.3%) although statistical analysis did not show that this difference was significant (Fig. 5).

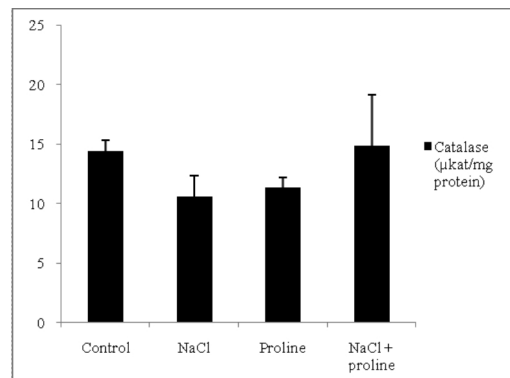


Figure 5. Effect of 20 mM exogenous proline application on catalase activity of durum wheat plantlets exposed to 10g.L-1 NaCl

DISCUSSION

The protective mechanism of exogenous proline in plants under NaCl stress has been increasingly reported in literature. This experiment showed different responses of physiological and biochemical parameters of durum wheat to the exogenous application of proline. In this study we have shown that the salt stress decreased the water content in plantlets compared to the control. This result has been also observed by Chorfi and Taibi [17] in Wheat. In response to exogenous proline, there was non-significant increase in the water content under salinity stress and, proline did not restore it to levels found in control plantlets. This observation is in conformity with the result obtained in tobacco by [53]. We can say that exogenously applied 20 mM proline could slightly maintain a higher water content under salt stress but it could not exhibit an ameliorative effect on water status of durum wheat plants subjected to NaCl stress at 10g/l. Boyer et al. [11] have showed that osmotic adjustment maintained turgor in durum wheat salinised to 150 mM NaCl, but measured leaf RWC decreased substantially. In tobacco, Okuma et al. [52] have found that 10mM of proline don't increase the relative water content in 100mM-NaCl-stressed plants. However, some authors have shown that the exogenous application of proline significantly ameliorated salt-stress induced inhibitory effects on plant water status on barley [71], maize [25] and cucumber [34] and in others species. Thus, our results do not support the hypothesis that the alleviation of the deleterious effects of salt by exogenous proline can be related to a protection of water statut in the leaves of plant. The result of salt stress on chlorophyll content in wheat is in agreement with those reported by [71] in barley, [47] in maize, [13] in rice and Atriplex [38]. It has been reported that membranes become unstable in response

to salt stress. This instability of membranes leads to reduction of leaf chlorophyll content as a result of NaCl-induced disruption of chloroplast integrity [51, 63]. In fact, during salinity, a part of glutamate is converted to proline that cause to decline input of this compound to chlorophyll biosynthesis pathway [7], therefore the chlorophyll content is reduced. In response to proline supplied externally under NaCl stress, the chlorophyll content of plantlets was positively affected compared to stressed plantlets which is consistent with the result of Raza [56] on wheat. These results are similar to those of some earlier reports on different plant species e.g, melon [40], maize [49], sunflower [36] and fennel [70]. It was attributed to the ability of proline to protect membranes against salt stress, to induce enzymes involved in pigments biosynthesis, and to stabilize protein complex (photosystem II) [40, 62]. Nawaz *et al.* [51] and Deivanai *et al.* [21] affirmed that the exogenous proline mitigates the detrimental effects of salt on chlorophyll pigment in sorghum and rice, respectively.

Contrary to what was expected, the addition of proline under saline conditions do not appear to have an effect on the sugar content of plantlets comparing to NaCl-stressed plantlets. One possible explanation for this is that in the presence of exogenous proline, sugars are not involved in the osmoprotection of wheat plantlets under salinity conditions. However, some authors have shown that the exogenous proline exhibit an increase of sugars on maize and broad bean [25] or a decrease on olive [1] under salt stress and osmotic stress respectively, suggesting the contribution of sugars in osmotic adjustment or the role of added proline to limit the need of salt-stressed plants for soluble sugars synthesis. The difference of plant species or concentrations of proline application may explain this inconsistency. The significant increase of the soluble proteins content of salt stressed plantlets by the exogenous proline is proposed to be due to proline action as a protectant of enzymes and proteins from damage and denaturation thanks to its hydrophobic property. Several previous studies on cereals and others species have shown the protective role of exogenous addition of proline on proteins [41, 37, 21]. It can be the result of the enhanced *de novo* synthesis of proteins for cell protection [66], in addition to its beneficial effect in stabilizing membranes and the protein synthesis machinery [41] and to a reduced membrane lipid oxidation [53].

In the other hand, under saline conditions no significant stimulation of proline accumulation on wheat was observed in response to the exogenous application of proline. We suggest that in wheat, proline has probably a role in the induction of osmolytes synthesis other than sugars and proline ones. This finding is inconsistent with the result of several studies which have demonstrated that exogenous proline was effective in accumulating the endogenous proline of salt-stressed plants. This accumulation has been attributed to its contribution to osmotic

adjustment directly by increasing the internal proline content [41]. In the absence of stress, the exogenous proline can activate the expression of proline dehydrogenase gene (*Prodh*) [42, 55] which is involved in proline oxidation and plays an important role in the regulation of proline levels in plants [69]. Thus, the proline content in plantlets was decreased by proline itself as compared to control. Like proline, glycine betaine is known as a compatible osmolyte. Our result shown that the salt stress increased the foliar glycine betaine content of wheat plantlets compared to the control. Interestingly, exogenous applied proline led to a remarkable increase in the accumulation of glycine betaine in saline conditions. This accumulation can be explained by the induction of genes encoding enzymes involved in glycine betaine biosynthesis by the exogenous proline suggesting the absence of an inhibitory effect between these two osmolytes. Similar result has been observed in maize by [3] under drought stress. It seems reasonable to suggest that the glycinebetaine accumulation participated in the stabilization of proteins, membranes and chlorophyll pigments of durum wheat against destabilizing effects of salinity. Chen and Murata [15] reported that glycine betaine effectively stabilizes the quaternary structures of enzymes and complex proteins, and it maintains the highly ordered state of membranes at non-physiological salt concentrations to provide a tolerance to cells.

The amount of malondialdehyde (MDA) often tends to show a greater accumulation under salt stress [72]. It is one of the byproducts and could reflect the degree of the peroxidation of membrane lipid and membrane un-stability associated with oxidative stress [34, 71]. Our results showed an increased level of MDA in stressed plantlets compared to non-stress conditions indicating that the NaCl stress caused oxidative damage to wheat plant. In the other hand, the proline treatment has substantially decreased the MDA content under stressed conditions. Additionally, we noted a significant reduction of the amount of total electrolyte leakage in salt-stressed plants by the exogenous proline suggesting a more stability of wheat-cellular membranes against salinity stress. Zeid [71] also observed a considerable decrease in electrolytes leakage in salt-stressed barley. These findings support the protective role of proline against oxidative stress induced by salt stress. Chen and Li [16] reported that proline may serve as a membrane stabilizer to block the attack of reactive oxygen species or ROS on cell membranes.

Generally, the ROS are scavenged by a variety of antioxidant defense systems that prevent ROS from reaching toxic levels [15]. The major ROS scavenging antioxidant enzymes include catalase [37, 14]. We observed that the catalase activity decreased under stressful conditions. This agrees with the results of some earlier reports in salt-sensitive species [9, 5, 4]. Such a decrease can be associated with the gene regulation of catalase biosynthesis or due to some

stress-induced injury to the enzyme. Based on a low RWC, NaCl-induced stress was severe and, in this case, the antioxidant activity was reduced. The effect of exogenous proline on catalase activity remained statistically non-significant. Similar results have been found by Zeid [71] and Reza et al. [57] in barley. Thus, our result may support the hypothesis of [41] that proline is able to maintain the catalase activity under stress compared to the control, since it can work as an enzyme protectant under stress. Among the ROS-scavenging antioxidant defence genes studied, Banu *et al.* [10] have found that mRNA levels of SAbind CAT (*salicylic acid binding catalase*) was increased by exogenous proline in cultured tobacco cells under 200mM NaCl stress. Further, Khedr *et al.* [41] have suggested that the peroxidase activity POD an other enzyme involved in the antioxidant system, may be more stable or more important for stress tolerance than catalase probably because of the wide range of metabolic processes in which this enzyme is known to be involved in addition to anti-oxidative activity [34]. Cruz de Carvalho (2008) [20] reported that the behavior of antioxidant enzyme don't only depend on the severity and duration of the stress treatment, but it also depends on the species and age of the plant.

In general, this study is the first contribution to understand the physiological and biochemical events occurring in durum wheat seedlings, confronted to salt constraint, further to the externally applied proline. Our findings indicated that the adverse effects of salt stress on durum wheat plantlets can be alleviated with the exogenous application of proline, at 20 mM level, by stabilizing sub-cellular structures, such as membranes and proteins. Further investigations are needed to ameliorate our understanding of the effect of exogenous proline on the NaCl tolerance of durum wheat.

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