# EFFECTS OF PROLINE ON MAIZE EMBRYOS CULTURED IN SALT STRESS

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## ABSTRACT

Effects of NaCl (50, 100 and 150 mM), proline (2.5, 5 and 10 mM), and their combinations on growth and contents of chlorophyll, proline, soluble protein and polyphenol oxidase (PPO E.C. 1.10.3.1) activity of maize (*Zea mays* cv. Merit) seedlings cultured *in vitro* were investigated. NaCl decreased seedling growth, fresh weight and chlorophyll content, while soluble protein and proline contents were increased. Proline added to control seedlings decreased root length and fresh weight, but did not change shoot length. Also proline increased chlorophyll, soluble protein and proline contents. When proline was added into the medium of NaCl-treated seedlings, growth, chlorophyll, soluble protein and proline contents were increased, in comparison to seedlings treated only with NaCl. PPO activity was increased by 5 mM proline treatment in all tissues of the maize seedlings growing in the salt medium. The results suggest that proline may play a role as an enzyme protector under conditions of salt stress.

**KEYWORDS:** embryo culture, chlorophyll, maize, polyphenol oxidase, proline, salt, soluble protein.

## INTRODUCTION

Salinity is one of the major stresses, which reduces plant growth and yield. Plants are stressed in two ways when exposed to conditions of high salt: 1. by an increased osmotic potential of the rooting medium in response to a high solute content, and 2. by the toxic effects of high concentrations of the ions. Salt stress results in a wide variety of physiological and biochemical changes in plants. Among these are accumulation of low-molecular-weight solutes, such as proline, commonly referred to as compatible solutes, and changes in free polyamine levels. The physiological significance of these responses in higher plants is a matter of controversy, because direct evidence for the role of proline or polyamines during acclimation to stress conditions remains tenuous [1].

Proline accumulation in plants subjected to salt stress has been indicated in several reports [2]. There are also several reports on the role of proline in plant salt tolerance. Many researchers have ascribed a positive role to proline under stress conditions. Possible functions of proline include maintaining osmoregulation [3], stabilizing proteins [4], and protecting cell membrane and cytoplasmic enzymes [5]. In light of the above, proline accumulation has even been suggested as a measure of salt tolerance [6]. In comparison, some researchers claim that proline accumulation is a coincidental result of metabolic irregularities created by salt stress, and therefore it has no adaptive value [7-8]. Hanson et al. [9] considered proline accumulation to be a symptom of damage. According to Stewart and Lee [10], proline is an osmotic agent. Other researchers have suggested that proline degradation provides a source of energy, and carbon and nitrogen sources for the facilitated recovery of tissues following stress conditions [11]. As well, proline is considered to play an important role in the defense mechanisms of stressed cells, which can ameliorate shifts in the redox potential by replenishing the $NADP^+$ supply. Thus, the physiological role of proline in plants under salt stress is still uncertain.

To date, studies on salinity have focused predominantly on germination percentage and growth of seedlings and plants. There are several studies on the effect of salinity on the enzymes involved in seed reserve mobilization, such as amylase [12], protease [13], and RNAse [14]. A number of oxidation systems other than the major oxidative phosphorylation chain within the mitochondria have been reported in seeds and seedlings. Typically, these systems may either interfere with normal respiration, or they may play a role in the recycling of reducing equivalents. One important system, wherein some phenols are oxidized to quinones, involves polyphenol oxidase [15]. This enzyme has been studied in a number of plants in relation to diseases, wounding [16], hormonal regulation [17], enzymatic browning [18], and germination [19]. However, data concerning its modulation under salt stress is lacking.

We have not encountered any report on the response of PPO to NaCl and proline in maize seedlings. Therefore,

the aim of this study was to research the effect of proline on the growth of maize seedlings cultured *in vitro* under saline conditions with respect to some biochemical parameters, such as chlorophyll and protein contents as well as PPO activity.

## MATERIALS AND METHODS

### Plant material and growth conditions

Seeds of maize (*Zea mays* cv. Merit) were surface-sterilized by soaking in 1 % sodium-hypochloride for 10 min and washed with distilled water. Embryos of the seeds were excised and transferred into glass jars containing sterile Murashige and Skoog (1962 MS) medium, without hormones. The pH of the medium was adjusted to 5.7 with KOH. Agar (0.6 %) and sucrose (3 %) were added. This basal nutrient medium was modified by the addition of NaCl (50, 100 and 150 mM), proline (2.5, 5 and 10 mM), or a combination of them. The basal medium was used as control. The embryos were grown for 7 days in a growth chamber (temperature of 24 $^{o}$C, photoperiod 14 h, irradiance of 222 µmol $m^{-2}$ $s^{-1}$ (400-700 nm) photon flux density). After 7 days, plants were harvested and lengths and fresh masses of roots and shoots were recorded.

### Determination of chlorophyll content

Chlorophyll (Chl) was extracted with 80% acetone from leaves after sedimentation by centrifugation at 6,000 rpm for 15 min. Absorbance of the extracts was measured spectrophotometrically (Shimadzu UV 1208, Tokyo, Japan) at 645 and 663 nm. Arnon's formula [20] was used for the estimation of chlorophyll a, b and total chlorophyll.

### Determination of soluble protein content

Quantitative protein determination of the tissues was carried out spectrophotometrically at 595 nm according to Bradford's method [21] after extraction in 0.05 M phosphate buffer (pH 6.5), with bovine serum albumin as a standard.

### Determination of proline content

0.5g of plant material was homogenized in 10 ml of 3% aqueous sulphosalicylic acid (Sigma St. Louis, MO, U.S.A.) and the homogenate was filtered through a Whatman #2 filter paper. These filtrates typically reacted with an acid-ninhydrin solution, which was prepared by warming 1.25g ninhydrin in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid, with agitation, until dissolved. Stored at 4 $^{o}$C, the reagent remains stable for 24 h. Two-milliliter samples were incubated with 2 ml of reagent solution in a test tube for 1 h at 100 $^{o}$C, and the reaction was terminated in an ice-bath. The reaction mixture was extracted with 4 ml toluene and vortexed vigorously for 15-20 seconds in a test tube. The toluene-solubilized chromophore (upper phase) was separated from the aqueous phase via aspiration, warmed to room temperature, and absorbance was read at 520 nm against pure toluene as a zero blank. The proline concentration was determined from a standard curve and calculated on a fresh weight basis as follows:

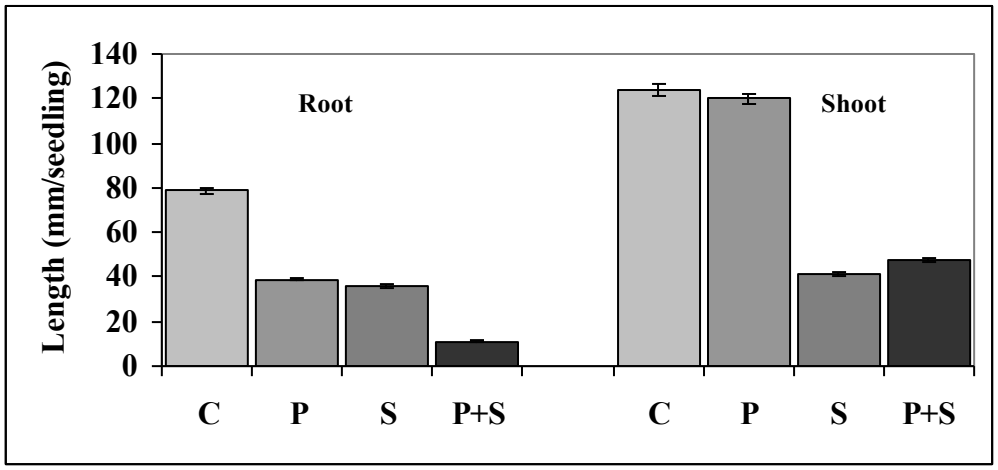
[(µg proline/ml x ml toluene) / 115.5 µg/µmole]/ [(g samples)/5] = µmoles proline/g of fresh weight material [22].

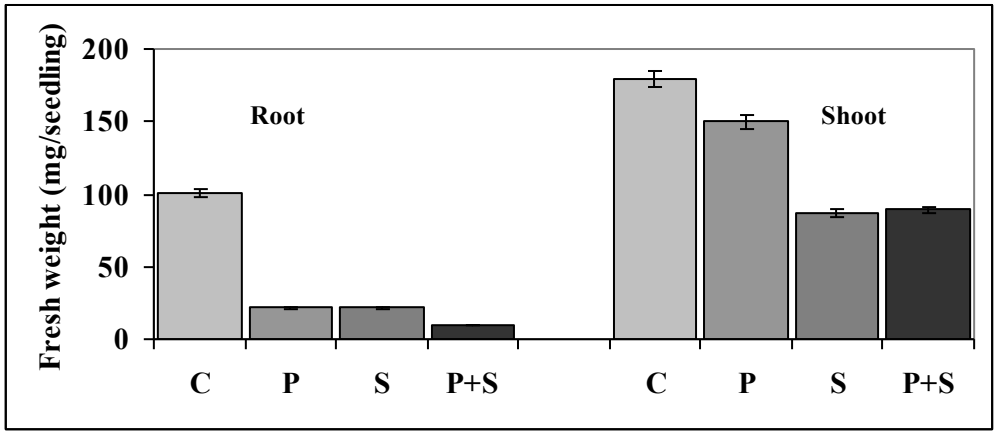
### Determination of PPO activity

Fresh tissue samples (0.5 g) from root, stem and leaf of the seedlings were homogenized with 5 $cm^3$ cold 0.05 M sodium phosphate buffer (pH 6.5) for 2 min and filtered, before being centrifuged at 20,000g for 15 min at 4 $^{o}$C. Then, the enzyme activities were immediately determined spectrophotometrically as described by Jennings and Duffus [18]. 3,4-Dihydroxyphenylalanine (DOPA; 10 mM), prepared in 0.1 M (pH 6.5) sodium phosphate buffer, was used as a substrate. The reaction mixture containing 2.8 $cm^3$ of substrate solution and 0.2 $cm^3$ of crude extract was incubated at room temperature for 2 min. The absorbances of the mixture were measured spectrophotometrically (Shimadzu UV 1208 spectrophotometer) at 420 nm. The mixture without crude extract served as a blank.

## RESULTS AND DISCUSSION

All glycophytes showed a reduction in growth, with regard to NaCl-induced stress. Lowered water potential and impairment in metabolic processes by NaCl are ultimately responsible for the inhibition of the overall growth [23]. In this study, growth was greatly affected, since root-shoot lengths and fresh weight decreased parallel with the salt concentrations. Salt has a more negative effect on root than shoot (Fig. 1). Chlorophyll a and b contents were greatly reduced with decreasing water potential of the growth medium by the addition of salt (Fig. 1). Similar results reported that growth, chlorophyll content and mineral uptake are significantly reduced under abiotic stress conditions [2, 24, 25]. NaCl increased the soluble protein contents in the root, stem and leaf tissues (Fig. 2). Increasing soluble protein content induced by NaCl has been reported previously [26, 27], and proline addition has been found to increase the soluble protein content in saline medium [28]. Similar trends have been reported previously in the sense that, with increasing NaCl concentrations, the photosynthetic rate decreased and fresh mass and root length of the regenerates declined. NaCl also induced the formation of new proteins [29]. In the present study, proline application caused reductions of root-shoot lengths and fresh weight parallel to the proline concentrations and increased the chlorophyll, protein and proline content. Proline displayed a more negative effect on root than on shoot development. The negative effect of NaCl on elongation and fresh mass of shoot in the maize seedlings was alleviated in the combination of 150 mM NaCl + 10 mM proline (Fig. 1). Other trials (50, 100 mM NaCl and 2.5 and 5 mM proline) were not presented herein, because they did not show a similar positive effect under salt stress conditions.



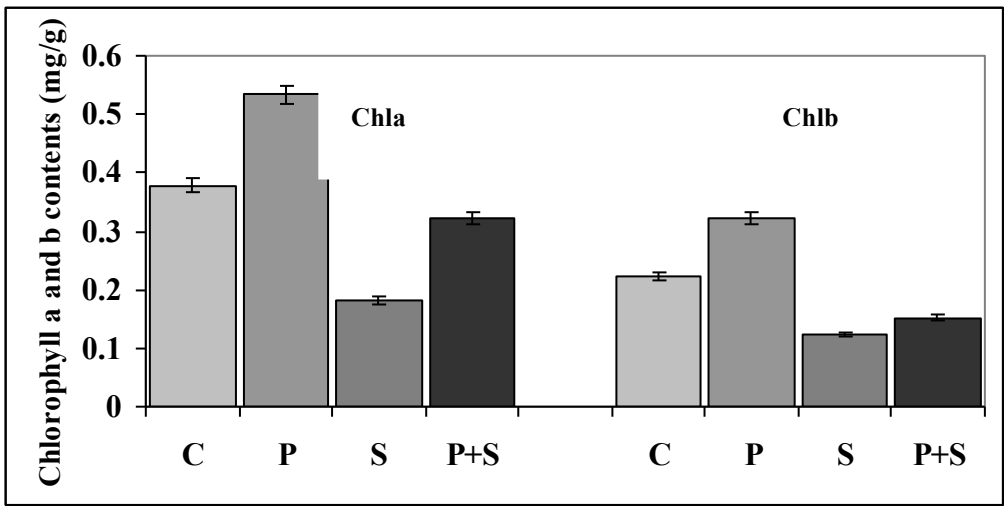




**FIGURE 1 - Effect of 10 mM proline (P), and 150 mM NaCl (S), and their combination (P+S) on length, fresh mass and chlorophyll $_{a+b}$ contents of maize seedlings. Presented is the mean ± standard error for triplicate samples. Bars represent SE. (C- control).**

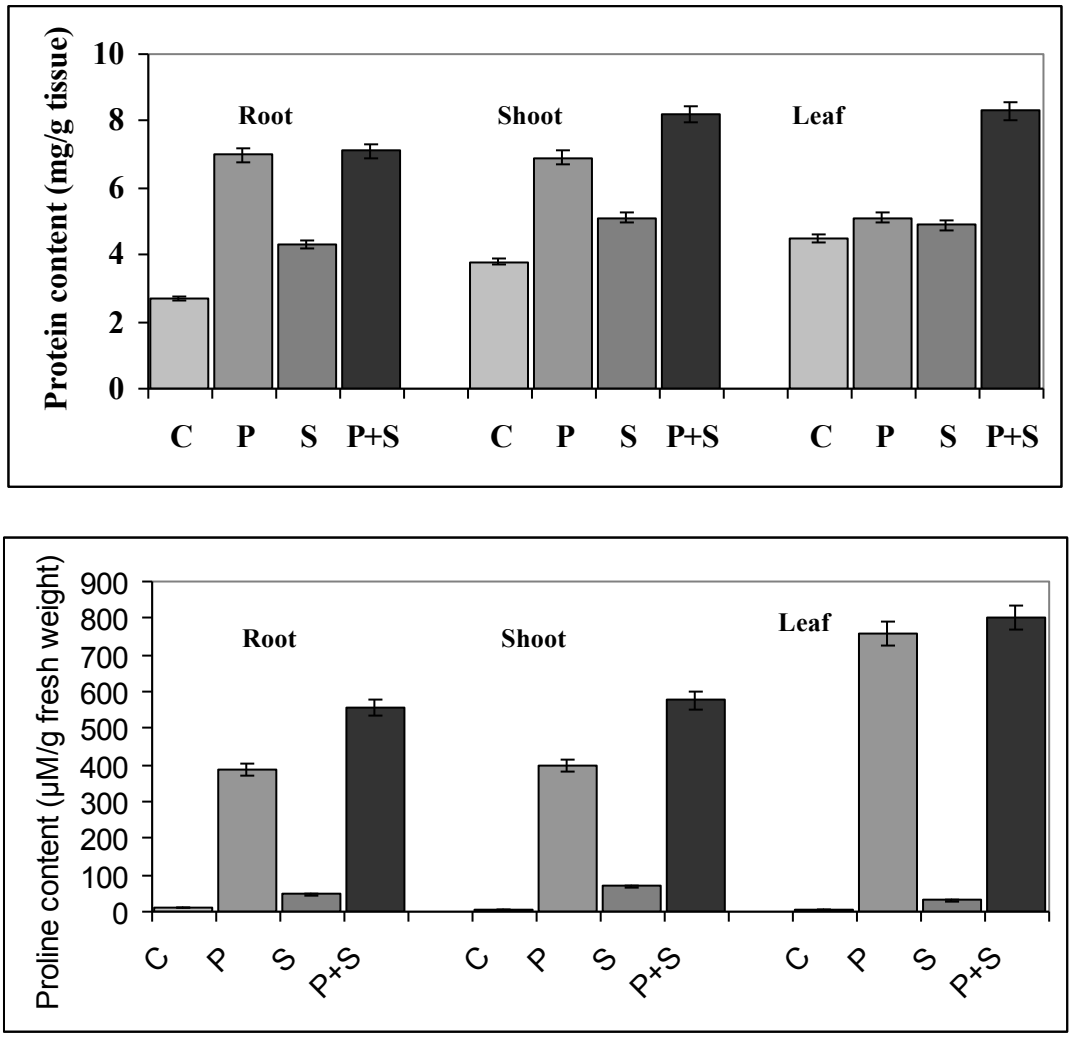



**FIGURE 2 - Effect of 10 mM proline (P), and 150 mM NaCl (S), and their combination (P+S) on protein and proline contents in maize seedlings. Presented is the mean ± standard error for triplicate samples. Bars represent SE. (C- control).**

Organisms tolerate abiotic stresses by accumulating a variety of cytosolutes, termed compatible solutes [30]. Proline is one of the compatible solutes, which accumulates substantially in stressed plants [31]. Proline, sugars and sucrose facilitate osmotic adjustment during stress [32, 33], and protect the native structure of macromolecules and membranes during extreme dehydration [34, 35]. Consequently, proline represents an important parameter to measure the stress tolerance capacity of the plants [31]. In our study, the endogenous proline and soluble protein contents of the maize seedlings were also increased by NaCl or proline treatments (Fig. 2). Similar trends have been reported in cases where exogenously applied proline has been easily absorbed by roots and transported to stem and leave [36]. NaCl + proline combination ameliorated reductions of root-shoot lengths and fresh weight, and increased chlorophyll, protein and proline content in salt media (Figs. 1 and 2). However, proline was seen to overcome salinity stress at 10 mM concentration. Lower concentrations of proline (2.5 and 5.0 mM; data not shown) were also examined in this study, but these concentrations were inadequate to overcome the effects of salt stress. One mechanism of salt tolerance involves an osmotic adjustment [37] that implies a net increase of solutes, a compartmentation of $Na^+$ and $Cl^-$ ions inside the vacuole, and an accumulation of organic solutes in the cytosol. Among these solutes, proline may constitute 50% or more of the amino acid fraction, and 10-20% of the dry matter in several halophytes [38].

PPO is one of the major enzymes responsible for oxidation of phenolic compounds [39]. PPO activities had not changed markedly in any tissues treated with NaCl, when compared against control tissues. In 50 mM NaCl medium, 5 mM proline concentration increased the PPO activity, whereas 10 mM proline did not alter the activity, in comparison to controls in all tissues. In 100 mM NaCl medium, 5 and 10 mM proline concentrations did not change

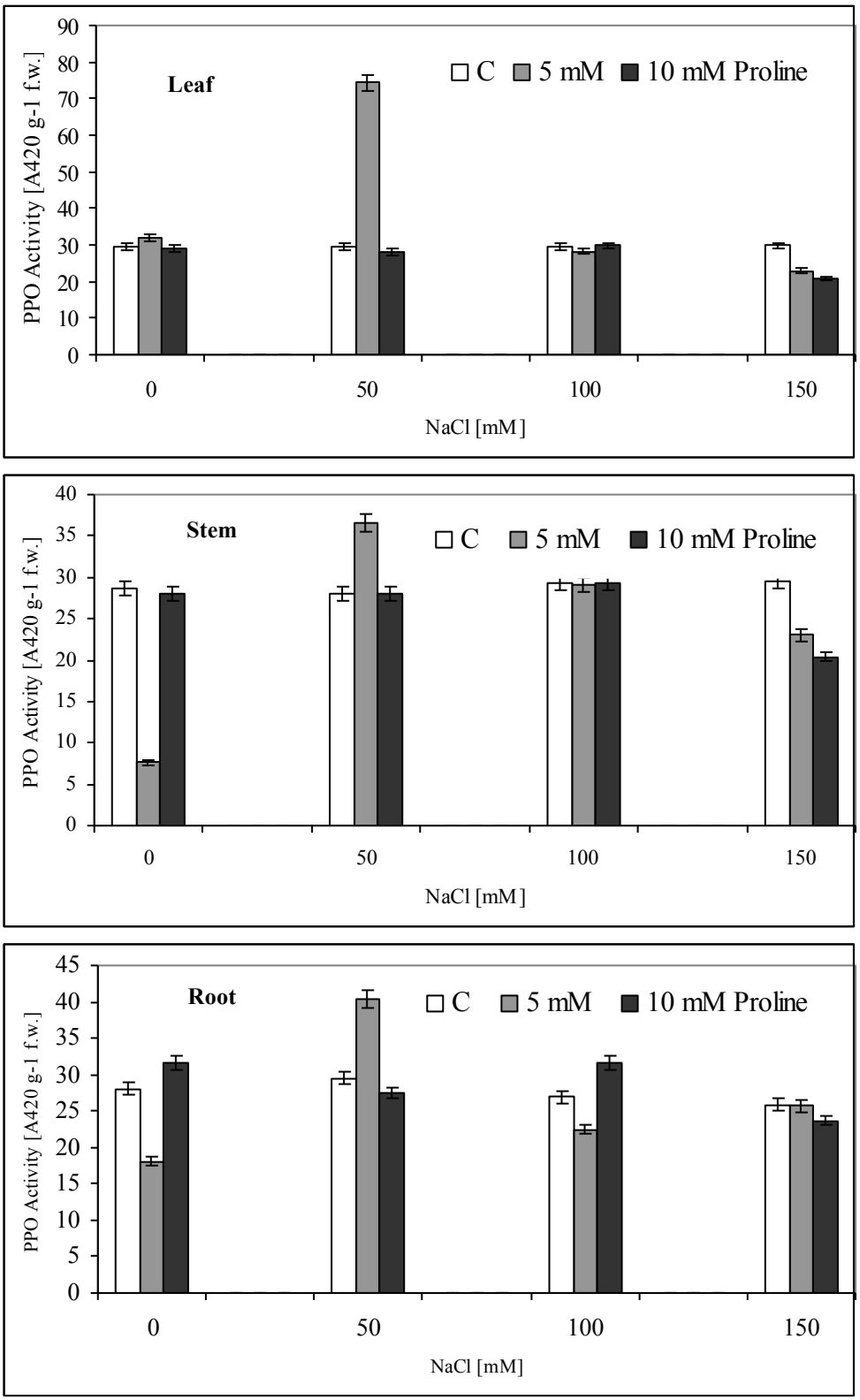



**FIGURE 3 - Effect of NaCl and proline in different concentrations on PPO activities in root, stem and leaf tissues of 7-days old maize seedlings grown in embryo culture. Presented is the mean ± standard error for triplicate samples. Bars represent SE. (C- control).**

the activity. In 150 mM NaCl medium, 5 and 10 mM proline concentrations decreased the PPO activity with respect to controls in all tissues (Fig. 3). In another study conducted on spinach, the PPO activity was increased by the exogenous application of praline, whereas it was decreased by NaCl. PPO activities were also increased by the addition of proline + NaCl [40]. In the present study, 5 mM proline treatment increased PPO activity in all the tissues of the maize seedlings growing in the presence of 50 mM NaCl (Fig. 3). This result is in agreement with previous ones [41-43], indicating that proline protects enzymes under saline conditions of the salt stress. This protection may be in the form of some structural modulations which keep them active under salt stress, or may be protecting the enzymes against proteolytic degradation.

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