Journal of Applied Biology & Biotechnology Vol. 1 (03), pp. 011-020, October, 2013
Available online at http://www.jabonline.in
DOI: 10.7324/JABB.2013.1302


# Role of calcium in increasing tolerance of Hyacinth bean to salinity

Myrene R. D'souza[1*] and Devaraj V. R.[2]

[1]Dept. of Chemistry, Mount Carmel College, Vasanthanagar, Bangalore– 560 052, India.
[2]Dept. of Biochemistry, Central College Campus, Bangalore University, Bangalore-– 560 001, India.

**ARTICLE INFO**

*Article history:*
Received on: 21/09/2013
Revised on: 8/10/2013
Accepted on: 15/10/2013
Available online: 30/10/2013

***Key words:***
calcium chloride,
chlorophyll,
malondialdehyde,
peroxidase, proline, sugars.

**ABSTRACT**

In many crop species, supplemental Ca2+ alleviates the inhibition of growth in plants subjected to NaCl stress. This work assesses the ameliorating effect of CaCl2 on NaCl-stressed seedlings of Hyacinth bean. Ten day old seedlings were stressed either solely with NaCl (100-500 mM NaCl) or with NaCl (100-500 mM NaCl) + CaCl2 (10 mM) and compared with control and CaCl2 (10 mM) treated seedlings. Seedlings were harvested at time intervals of 24, 48 and 72 h after stress for analysis. NaCl-stressed seedlings showed reduced growth as indicated by growth index and relative water content (RWC) while a comparatively less decline in these parameters was seen in seedlings stressed with supplemental CaCl2. An enhancement in levels of H2O2, MDA, GSH, ASC, TSS and photosynthetic pigments noted in stressed seedling supplemented with CaCl2 was evident of its role in ameliorating salinity stress. Supplementation was also found to increase the activity of metabolic enzyme AMY paving the way for partial amelioration of stress caused by salinity.

## 1. INTRODUCTION

The progressive natural and anthropogenic salinization of arable lands at the rate of three hectares per minute worldwide [1] is a major concern for agricultural crop production [2]. The sustainability of agriculture production in many areas of the world including North and South America, Asia, Europe and Australia is at risk due to soil salinization [3-5].

Salinization is known to be associated with large scale irrigation practices. In the Indian subcontinent, the construction of large irrigation canals was said to have initiated the process of secondary salinization in several regions. An example of this is the emergence of salinity in the Deccan Plateau, with the commissioning of the Nira Irrigation Project in Maharashtra [6]. Salinity stress is generally caused by osmotic stress and ionic imbalance, the later effecting plants experiencing salinity for a longer duration [7].

Plants respond to salt stress by a complex interplay of proteins and genes which is displayed through morphological, physiological and biochemical changes. Only a few plants can successfully tolerate salt stress and complete their life cycle. Most plants are unable to do so and succumb to the stressful conditions leading to drastic agricultural losses.

* *Corresponding Author*
*Mail id: myrene83@gmail.com*

This difference in response to salt and other stresses is due to spatial and temporal variations in the relative amounts of compatible solutes, antioxidants, antioxidant enzymes and other stress-proteins. The enzymes in living cells have evolved to function at very low ionic concentrations, mainly 100-200 mM $K^+$ and less than 50 mM $Na^+$ and $Cl^-$. In addition, the homeostasis of not only $Na^+$ and $Cl^-$ but also of essential cations such as $K^+$ and $Ca^{2+}$ is disturbed [8,9]. Many researchers have thus recommended the application of supplemental doses of $Ca^{2+}$ to ameliorate stress symptoms [10, 11]. $Ca^{2+}$ ion, in optimum concentration, plays crucial roles in different physiological processes of plants and can cause increase in plant resistance to abiotic stresses [12].

In addition, it has been noted that $Ca^{2+}$ participates in the regulatory mechanism of plants' adjustment to adverse conditions; such as high temperature [13], cold injury [14], drought stress [15], and salt stress [16,17]. It has been reported that high extracellular $Na^+$: $Ca^{2+}$ ratio causes increased $Na^+$ influx [11].

Supplemental $Ca^{2+}$ acts as an inhibitor to many cell membrane channels such as KIRC ($K^+$ Inward Rectifying Channels) which reduces $Na^+$ influx under salinity stress [18], decreases $Na^+$ transfer to shoots [10], promotes absorption and maintenance of $K^+$ [19], causes activation of proline oxidase [20] and maintains structural integrity of amylase [21]. $Ca^{2+}$ was also found to replace water of hydration associated with the phosphate groups of membrane phospholipids, thereby enabling the plant to withstand

higher salt concentration in the soil [22]. Greenhouse and laboratory techniques are usually used for screening at the germination or early vegetative stages of growth. The seedling stage is the most sensitive phase of plant development and most of the research work involving the study of salt tolerance has been aimed at this phase of growth. Considering the reported role of calcium under abiotic stress and normal physiological conditions of plants, its effect on Hyacinth bean under salinity stress was investigated.

## 2. MATERIAL AND METHODS

### 2.1 Plant material

The seeds of Hyacinth bean, *Lablab purpureus* (cv. HA-4) were procured from National Seed Project, University of Agricultural Science, GKVK, Bangalore, India. Uniform sized seeds of *Lablab purpureus* cultivar HA-4 were surface sterilized with 0.1% (w/v) mercuric chloride for 1 min followed by three rinses in sterile distilled water.

### 2.2 Salt stress treatment

Overnight-soaked seeds were sown in plastic trays containing vermiculite and acid-washed sand (1:1 w/w) and irrigated daily with distilled water. The germination was carried out under greenhouse conditions; day/night temperature and relative humidity were 30/25 ºC, and 75/70 %, respectively. The average photoperiod was 12 h light/12 h dark.

Ten days old seedlings of uniform size were treated as follows; (1) For salt treatment, seedlings were fed with 0.5X Hoagland medium [23] containing 100 - 500 mM NaCl. (2) For salt treatment in the presence of $CaCl_2$, seedlings were fed with half-strength Hoagland medium containing 10 mM $CaCl_2$ combined with the above levels of NaCl. Leaf and root samples were collected at 24, 48 and 72 h, and frozen until further analysis. Plants grown on 0.5X Hoagland media without NaCl (C), and media with 10 mM $CaCl_2$ (Ca-C) served as control. Samples used for determination of RWC, fresh and dry weight were used immediately after collection.

The experimental design used was carried out at random factorial scheme, with 12 media regimes [Controls (C, Ca-C), 100 – 500 mM NaCl, 100 – 500 mM NaCl (in the presence of 10 mM $CaCl_2$)] and 3 evaluation points (24, 48, 72 h). Each experiment consisted of 72 experimental units (leaf + root samples) and was done in triplicate.

### 2.3 Determination of growth index

To investigate the recovery of the stressed plants after re-watering with 0.5X Hoagland medium, growth of seedlings i.e., the length of roots was measured after 3 days. The results for each variant were calculated from 20 seedling measurements and presented as the growth index (% control) expressed as the proportion of the length of roots from the stressed seedlings in relation to the same parameter from the control seedlings. Each treatment was replicated thrice. The whole experiment was performed in darkness, at 25 °C and humidity of 60-65 %.

### 2.4 Determination of metal content

Shoots and roots of $CaCl_2$- and NaCl-treated plants were oven-dried at 70 ºC to constant weight. The dry samples were ground into a fine powder and assayed for minerals using the wet digestion method [24].

The ion contents ($Na^+$, $Ca^{2+}$) were assayed using an atomic absorption flame photometer (AA-6601 F; Shimadzu Corporation, Tokyo) essentially according to Weimberg [25].

### 2.5 Relative Water Content (RWC)

The relative water content was estimated according to the method of Turner and Kramer [26] using the equation: RWC = (FW-DW) X 100 / (TW-DW). Leaf discs of 10 mm diameter were weighed to determine the fresh weight (FW), soaked in distilled water at 25 ºC for 4 h to determine the turgid weight (TW), then oven dried at 80 ºC for 24 h to determine the dry weight (DW). Similarly, entire shoot and root was taken for analysis and RWC was computed as before.

### 2.6 Determination of $H_2O_2$ and antioxidants

*Hydrogen peroxide:* $H_2O_2$ content in control and stressed seedlings were determined according to Velikova [27].
*Ascorbic acid (ASC):* Ascorbic acid estimation was carried out according to the procedure of Sadasivam and Manickam [28].
*Glutathione (GSH):* GSH was estimated according to Beutler [29].

### 2.7 Determination of stress response factors

#### *27.1.Proline*

The estimation was carried out according to the method of Bates [30].

#### *2.7.2.Malondialdehyde(MDA)*

The extent of lipid peroxidation was determined according to Heath and Packer [31].

#### *2.7.3.Photosynthetic pigments*

The procedure of chlorophyll determination was based on the work of Mackinney [32] on the absorption of light by aqueous acetone (80%) extracts of chlorophyll with at 663 and 645 nm. The concentrations of total chlorophyll, chlorophyll-a, -b and total carotenoids were calculated as given below:

Chl a (mg $g^{-1}$) = $[(12.7 \times A_{663}) - (2.6 \times A_{645})] \times (V / 1000 \times wt)$
Chl b (mg $g^{-1}$) = $[(22.9 \times A_{645}) - (4.68 \times A_{663})] \times (V / 1000 \times wt)$
Total Chl = $[(20.2 \times A_{645}) + (8.02 \times A_{663})] \times (V / 1000 \times wt)$
Carotenoids = $A_{480} + (0.14 \times A_{663} – 0.638 \times A_{645})$

The chlorophyll stability index (CSI) was calculated according to the method of Murty and Majumdar [33] as the ratio between Chl content in stressed leaves and Chl content in control leaves and expressed in %.

***2.7.4.Total soluble sugars (TSS)***

Frozen samples (0.5 g) were ground and extracted in 2 ml of 2.5 N HCl for 30 min, followed by centrifugation at 10,000×g at 4 °C for 10 min. Total soluble sugar content was estimated colorimetrically using anthrone reagent and glucose as standard [34]. Results are expressed as mg soluble sugar/g FW tissue.

**2.8 Activities of Metabolic enzymes**

***2.8.1.Enzyme extraction***

The frozen samples were homogenized with pre-chilled 50 mM sodium phosphate buffer (pH 7.0) containing 5 mM β-mercaptoethanol and 1mM EDTA using pestle and mortar. The homogenate was centrifuged at 12,000g for 15 min at 4 °C. The supernatant was used as a source of enzymes. Soluble protein content was determined according to the method of Lowry et al. [35] with BSA as the standard.

**2.8.2.β-*Amylase (AMY, EC 3.2.1.1)***

Activity of β-AMY was measured using the DNS method [36]. The reaction mixture consisted 0.5 ml of 2% starch solution dissolved in 50 mM phosphate buffer (pH 7.0) and 0.5 ml of enzyme extract.

***2.8.3.Invertase (INV, EC 3.2.1.26)***

INV activity was determined by the method of Sridhar and Ou [37]. 4.0 ml reaction mixture containing 0.025 M sodium acetate buffer (pH 5.0), 0.625 % sucrose, and appropriate volume of enzyme extract was incubated at 37 °C for 24 h. The reaction was arrested by adding equal volume of DNS reagent. The reducing sugars present were estimated using the method of Miller [38].

**2.9 Statistical analysis**

The experiment was performed using a randomized design. All data are expressed as means of triplicate experiments unless mentioned otherwise. Comparisons of means were performed using PrismGraph version 3.02. Data were subjected to a one-way analysis of variance (ANOVA), and the mean differences were compared by lowest standard deviations (LSD) test. Comparisons with $P \leq 0.05$ were considered significantly different.

**3. RESULTS AND DISCUSSION**

**3.1.Effect of stress treatments on germination index, RWC, and mineral composition**

Saline soils are not found to favor good germination of seeds [39,40]. Germination of seeds of Hyacinth bean was significantly affected by NaCl stress even when followed by re-watering. Increasing amount of NaCl caused progressive reduction in germination. The extent of reduction in germination ranged from 18.6 % under 100 mM to 42.4% under 300 mM NaCl (Table 1). The inhibitory effect of NaCl on germination was partially relieved by incorporating 10 mM $CaCl_2$ along with NaCl.

Imposition of salt stress reduced the RWC in seedlings (Table 1). The RWC of shoots showed steep decline at all times of exposure up to 300 mM NaCl and did not change much beyond 400 mM NaCl. Seedlings treated with NaCl+$CaCl_2$ showed a slight increase in RWC compared with those stressed with NaCl (Table 1). A higher $Na^+$:$Ca^{2+}$ ratio is known to reduce hydraulic conductance to the root and growing regions of the shoot. Supplemental $Ca^{2+}$ was found to prevent the inhibition of hydraulic conductance in maize [41].

Plant growth in saline medium influences the concentration of $Na^+$ and $K^+$ in various tissues. In this study, the $Na^+$ content of shoots and roots increased significantly and progressively with rise in NaCl concentration; with maximum accumulation taking place in the roots, followed by leaves (Table 1). This preferential accumulation of $Na^+$ in roots enables the plant to sequester most of the toxic ion [42]. Exogenous $CaCl_2$ application reduced the $Na^+$ content, regardless of the salinization level or the plant organ analyzed. Contrary to $Na^+$, the concentration of $K^+$ decreased in all tissues with the rise of salinity level. However, treatment with NaCl+$CaCl_2$ led to a minor increase in $K^+$ (Table 1). The increase in $K^+$ content in plants treated with exogenous $CaCl_2$ is in agreement with the results obtained by Heikal and Shaddad [43] and Abd El-Samad [44]. Monovalent ions are also known to substantially reduce membrane associated $Ca^{2+}$ [45]. One consequence of this displacement is the immediate increase of $K^+$ efflux across the plasma membrane [46]. Under high external $Na^+$ concentration, $Na^+$ can inhibit $K^+$ uptake and enter the cell through potassium channels. $K^+$ discrimination over $Na^+$ is essential for acquisition of salt tolerance [47,48]. Increasing $Ca^{2+}$ improves salinity tolerance of crop plants by reducing the $Na^+$ content in tissues (Table 1). Physiological experiments have indicated that the effect is mediated through an increase in intracellular $Ca^{2+}$, changes in vacuolar pH, and activation of the vacuolar $Na^+/H^+$-antiporter [49].

**3.2.Response of antioxidants**

$H_2O_2$, a form of ROS produced as a result of oxidative stress is the most stable among all the intermediates of oxygen reduction. The salt stress in Hyacinth bean resulted in increased production of $H_2O_2$ in time- and concentration-dependent manner. Hyacinth bean exposed to NaCl at 300 and 400 mM NaCl for 48 and 72 h showed greater increase in $H_2O_2$ (Fig. 1a) in leaves and roots. A progressive $H_2O_2$ accumulation was seen in salt-treated rice [50] and Lemna [51]. This is mainly due to osmotic stress induced by the external NaCl concentration that serves as a signal to set off the defense system in different parts of the plant. Inclusion of $CaCl_2$ along with NaCl in the growth media also showed elevated levels of $H_2O_2$; however, the overall concentration was less than that produced by NaCl alone (Fig 1a).

Salinity stress alters the structure of membranes due to lipid peroxidation. The degradation of polyunsaturated fatty acids due to peroxidation produces peroxide ions and MDA leading to membrane rigidification and cell death. It has been associated with damage provoked by a variety of environmental stresses and is

often used as an indicator of salt-induced oxidative damage [52]. The increase in MDA content has been found to be associated with the increase in $H_2O_2$ levels [53]. Salt stress in Hyacinth bean showed greater elevation in MDA content with increasing time of exposure (Fig 1b), indicating increased lipid peroxidation with increasing concentration of NaCl and extended exposure. Treatment with exogenous $CaCl_2$ demonstrated lower MDA levels when compared to seedlings treated with only NaCl (Fig. 1b). Diminished MDA levels in the presence of $CaCl_2$ were reported in cucumber treated with exogenous $CaCl_2$ [39]. The impact of the oxidative stress depends on the interaction of several factors that determine the antioxidant status of the plant. Ascorbate (ASC), the key antioxidant in plants, reacts directly with hydroxyl radicals, superoxide and singlet oxygen [54]. ASC levels in leaves and roots of stressed seedlings increased progressively with exposure time and concentration (Fig 2b), indicating effective scavenging of ROS in Hyacinth bean. As a powerful reducing agent, ASC maintains chloroplastic α-tocopherol and metalloenzyme activity and acts as a reductant in enzymatic reactions and in nonenzymatic free radical scavenging of superoxide and $H_2O_2$ [55]. From these results, ASC appears to have a significant role in Hyacinth bean. The increase of GSH in leaf tissues, especially, under exogenous $CaCl_2$ (Fig 2a) is considered to be responsible for generating ascorbate via the ascorbate-GSH cycle. Parallel increase in ASC and GSH at extended exposure and very high concentration of salt in Hyacinth bean treated with $CaCl_2$ suggests their positive influence on ascorbate–glutathione cycle in leaves (Fig 2). GSH is actively involved in the cyclic transfer of reducing equivalents in the ascorbate/glutathione pathway, regulation of gene expression [56] and redox control of gene expression [57]. Roots, however, showed a decrease in GSH levels in all treated samples suggesting a minor role in salt tolerance compared to leaves. Lappartien et al. [58] have also reported similar observation with Canola.

### 3.3. Response of metabolic enzymes and soluble sugars

Induction of metabolic enzyme, β-AMY under abiotic stress has been reported [59]. Increased levels of β-AMY, as observed by in vitro levels (Fig 3) suggested induction of β-AMY in leaves of Hyacinth bean. The induction of β-AMY could be due to NaCl-induced osmotic signals, which in turn, could be linked to transitory starch degradation [60,61], causing an increase in maltose and total soluble sugars [62]. Increased β-AMY activity and corresponding increase in TSS were also seen in seedling stressed with NaCl+$CaCl_2$ (Fig 5a). Increased activity of β-AMY was also reported in *C. roseus* [63] and *Phyllanthus amarus* [64] treated with NaCl+$CaCl_2$. The binding of $Ca^{2+}$ to β-AMY is known to increase thermostability of the enzyme as well as reduce its susceptibility to proteolytic degradation [21].

TSS levels bear a direct correlation with proline biosynthesis and are related to acquisition of salt tolerance in plants [65]. In addition to their role in osmoprotection, TSS play an important role in biosynthetic processes, energy production, stabilization of cellular membranes, maintenance of turgor, and signaling [66]. Elevated levels of TSS in concentration-dependent manner during the first 24 h of exposure to NaCl stress suggested the probability of proline-mediated sugar biosynthesis in Hyacinth bean (Fig 4 a). However, inverse relationship between proline and TSS during prolonged exposure indicated no significant role for carbohydrates and/or carbohydrate metabolic enzymes in osmotic regulation. Treatment with NaCl+$CaCl_2$ was found to increase TSS levels in leaves even after 48 and 72 h of stress (Fig 4). However, a concentration- and time-dependent decline in TSS levels was noted in roots for all treatments especially after 48 h of stress (Fig 4a). This can be correlated with inhibition of β-AMY activity in roots (Fig 3). Since drought stress increased the concentration of TSS, it might be expected that activity of INV had been reduced. INV activity in both leaves and roots showed a clear decrease in response to increasing concentration of NaCl alone, and in combination NaCl+$CaCl_2$ (Fig 5). Decrease in INV activity along with other effects such as starch degradation, inhibition of starch synthesis and sequestration of photosynthates [4], explains the increase in TSS levels in leaves of Hyacinth bean. A marginal increase in TSS during first 24 h, a decline during further exposure in leaves, and a measurable increase during all the time points of exposure up to 300 mM NaCl in roots of Hyacinth bean indicated that osmotic homeostasis differs in leaves and roots. The results also indicated that sugar mediated osmotic regulation in leaves holds good only for short-term exposures.

### 3.4. Levels of other stress-response factors: Photosynthetic pigments and proline

ROS formation due to abiotic stress occurs primarily in the electron transport chains of mitochondria and chloroplasts. In thylakoids, ROS is produced due to over-reduction of PSI and PSII, which are no longer able to accept excess of excitation energy from light-harvesting chlorophyll protein complexes [LHCP] [67]. Such electron surplus that can lead to ROS formation can be avoided by degrading chlorophyll [68]. In the present study, the leaf chlorophyll content decreased while Chl a/b ratio increased (Tab. 2) suggesting that salinity injury may involve severe chlorophyll photooxidation mediated by oxy-radicals [69]. Such a decrease in chlorophyll content has been reported from salt-sensitive cotton [70] and *Phaseolus vulgaris* [71]. In addition to oxidative damage, increased chlorophyllase activity and salt-induced weakening of protein-pigment-lipid complexes have been implicated in chlorophyll degradation during stress conditions [72]. Thus, the observed reduction in chlorophyll content under NaCl stress could be a result of both decreased synthesis and increased degradation. Exogenous $CaCl_2$ however, did not prevent the degradation of chlorophyll induced by NaCl treatment. The increase in Chl a/b ratio seen under NaCl treatment (Table 2) is indicative of PSII/PSI content alterations that serve as a marker for abiotic stress [73]. Chl b is more sensitive to salt stress than Chl a [74] and hence causes an increase in Chl a/b ratio. Similar increase in Chl a/b ratio was reported in pearl millet subjected to salt stress [75]. The Chl a/b ratio in plants treated with exogenous $CaCl_2$ did not exhibit any such enhancement. The chlorophyll stability index (CSI), an indicator of the stress tolerance capacity of plants was

found to exhibit significant reduction under NaCl stress. Exogenous application of $CaCl_2$ did not exhibit any enhancement in CSI (Table 2). Many reports have emphasized the role of accessory pigments, carotenoids (especially xanthophylls) in direct deactivation of ROS [76,77]. Carotenoids are spatially associated with chlorophyll [67] and sustain photochemical functions by protecting chlorophyll against oxidative destruction [77]. The changes in the carotenoid content under different levels of salinity were parallel to those of chlorophyll pigment (Table 2). The response of carotenoid content in presence of exogenous $CaCl_2$ was similar to that produced by seedlings subjected to NaCl stress alone. Decrease in the carotenoid content at higher salinity levels has been reported to be due to degradation of β-carotene and formation of zeaxanthin in barley and sorghum [78]. The osmolyte, proline levels in shoots were elevated under salt stress (Fig 4b). The effect was moderate up to 300 mM during the entire period of exposure. Proline content showed greater improvements under longer duration of 48 h and 72 h and higher concentration of NaCl, i.e., 300 to 400 mM. However, at 500 mM NaCl, the proline levels showed a downward trend suggesting insufficiency of protective mechanism beyond 400 mM NaCl. This suggested the inherent ability of the protective system to produce osmo-protectant, proline in response to dehydration induced by NaCl. The importance of the oxidative pentose phosphate pathway (OPPP) in triggering seed germination is widely known. The link between OPPP and proline synthesis is also well established [79]. Increase in free proline content under NaCl stress is a general response to salinity stress by osmotic adjustment at cellular level [80]. Proline act as enzyme protectant and stabilizes the structure of macromolecules and organelles [81]. Increase in proline content under NaCl stress in this experiment was probably due to break-down of proline-rich protein or *denovo* synthesis of proline [80]. In the case of 10 mM $CaCl_2$ treatment, proline concentration was reduced when compared with those plants not treated with $CaCl_2$.

**Table. 1:** Effect of salt treatment on the growth index, RWC and mineral composition of Hyacinth bean supplemented with $CaCl_2$.

| Treat-ment | [NaCl] mM | Growth index (%) | | | RWC (%) | | | Mineral composition (mg/g FW tissue, 72h) Leaf | | | Root | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 24h | 48h | 72h | 24h | 48h | 72h | Na | K | Ca | Na | K | Ca |
| NaCl | 0 | 64.2 | 62.4 | 42.4 | 97.6 | 95.9 | 88.3 | 1.45 | 20.14 | 25.21 | 1.27 | 19.32 | 20.79 |
| | 100 | 69.3 | 71.1 | 81.1 | 82.6 | 68.5 | 71.9 | 2.63 | 19.11 | 25.04 | 3.34 | 18.10 | 20.44 |
| | 200 | 69.0 | 76.6 | 82.4 | 72.9 | 64.2 | 58.1 | 4.32 | 18.34 | 24.14 | 6.23 | 17.31 | 19.42 |
| | 300 | 45.2 | 52.9 | 57.4 | 66.6 | 73.3 | 53.5 | 12.3 | 15.13 | 26.37 | 15.7 | 12.42 | 21.75 |
| NaCl + $CaCl_2$ (10mM) | 0 | 69.2 | 69.2 | 41.4 | 88.3 | 89.3 | 88.3 | 1.31 | 21.44 | 28.94 | 1.34 | 20.42 | 27.41 |
| | 100 | 80.3 | 87.4 | 89.3 | 83.8 | 71.8 | 76.7 | 3.15 | 23.21 | 27.54 | 2.57 | 21.31 | 28.97 |
| | 200 | 80.3 | 87.4 | 89.3 | 74.4 | 70.4 | 66.9 | 5.66 | 24.29 | 29.43 | 5.21 | 18.42 | 28.97 |
| | 300 | 48.4 | 60.4 | 62.4 | 68.7 | 75.1 | 62.7 | 10.8 | 21.63 | 31.32 | 13.3 | 16.14 | 29.41 |

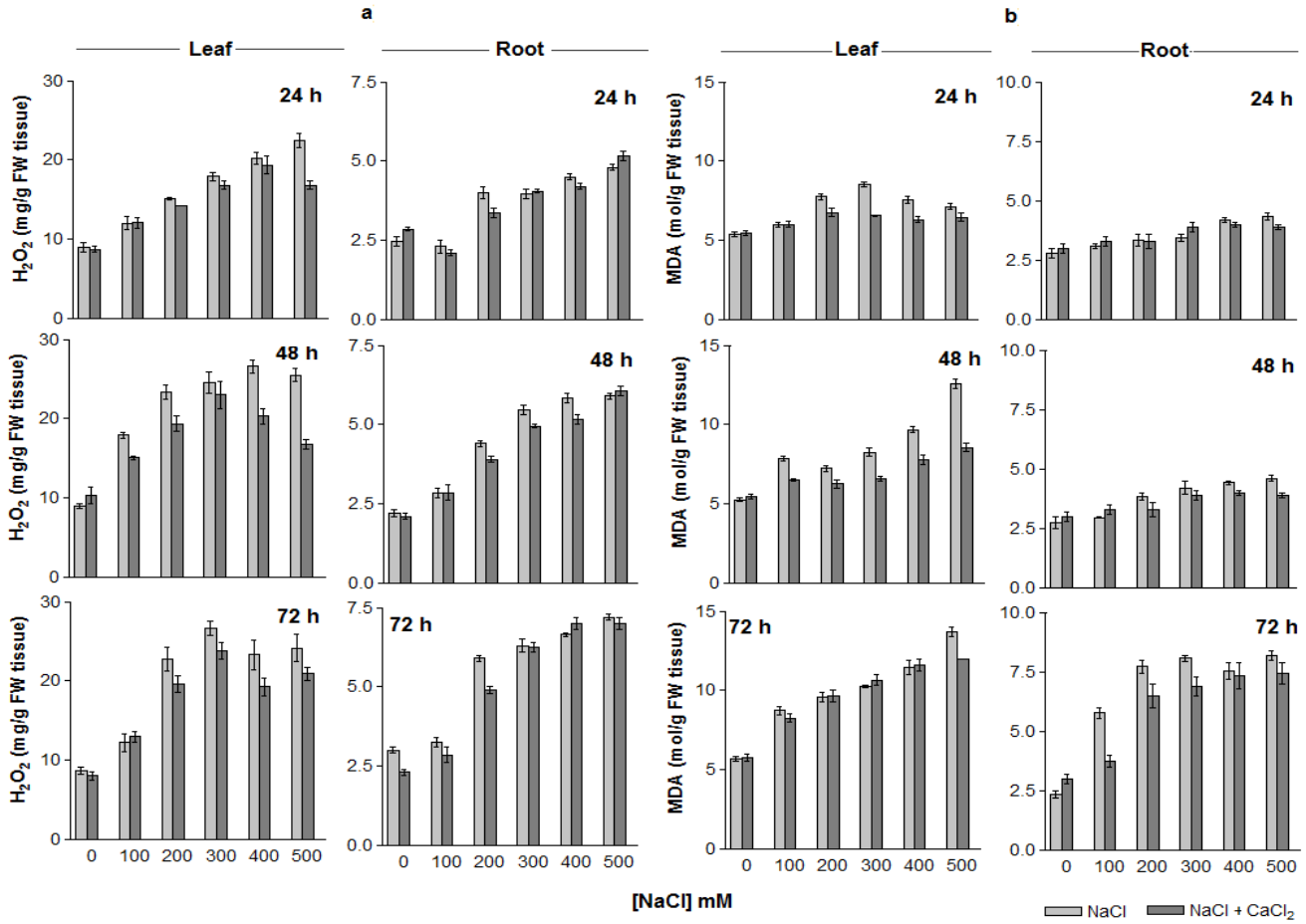


**Fig. 1**: Levels of $H_2O_2$ (a), malondialdehyde, MDA (b) in leaves and roots of Hyacinth bean supplemented with $CaCl_2$. Values are triplicates ± SE ($P \leq 0.05$).

**Table. 2:** Effect of salt treatment on photosynthetic pigments of Hyacinth bean supplemented with $CaCl_2$ expressed as mg/g of fresh weight tissue.

| Time | Sample | Total Chl | | Chl a/b | | CSI (%) | | Carotenoids | |
|---|---|---|---|---|---|---|---|---|---|
| | | NaCl | NaCl + Ca | NaCl | NaCl + Ca | NaCl | NaCl + Ca | NaCl | NaCl + Ca |
| 24 h | 0 | 8.81 ± 0.2 | 8.82 ± 0.3 | 2.08 ± 0.12 | 2.05 ± 0.1 | 100 | 100 | 0.59 ± 0.0 | 0.56 ± 0.0 |
| | 100 | 8.35 ± 0.4 | 7.90 ± 0.4 | 2.20 ± 0.1 | 2.25 ± 0.1 | 96.2 | 95.3 | 0.55 ± 0.0 | 0.53 ± 0.0 |
| | 200 | 7.96 ± 0.4 | 8.15 ± 0.6 | 2.32 ± 0.1 | 2.30 ± 0.1 | 92.8 | 91.4 | 0.71 ± 0.0 | 0.68 ± 0.0 |
| | 300 | 8.01 ± 0.4 | 8.05 ± 0.5 | 2.35 ± 0.1 | 2.15 ± 0.1 | 73.6 | 70.7 | 0.58 ± 0.0 | 0.56 ± 0.0 |
| | 400 | 7.71 ± 0.5 | 7.90 ± 0.6 | 2.58 ± 0.1 | 2.20 ± 0.0 | 80.3 | 79.4 | 0.50 ± 0.0 | 0.48 ± 0.0 |
| | 500 | 7.61 ± 0.5 | 7.65 ± 0.3 | 2.94 ±0.2 | 2.25 ±0.2 | 72.7 | 71.4 | 0.43 ± 0.0 | 0.42 ± 0.0 |
| 48 h | 0 | 11.2 ± 0.4 | 9.77 ± 0.2 | 1.71 ± 0.0 | 1.67 ± 0.1 | 100 | 100 | 0.64 ± 0.0 | 0.62 ± 0.0 |
| | 100 | 10.2 ± 0.5 | 8.70 ± 0.3 | 1.98 ± 0.1 | 1.92 ± 0.0 | 90.5 | 90.4 | 0.54 ± 0.0 | 0.56 ± 0.0 |
| | 200 | 9.55 ± 0.4 | 9.25 ± 0.4 | 2.14 ± 0.0 | 1.99 ± 0.1 | 82.7 | 83.1 | 0.59 ± 0.0 | 0.55 ± 0.0 |
| | 300 | 8.27 ± 0.6 | 8.20 ± 0.3 | 2.43 ± 0.1 | 2.05 ± 0.2 | 65.7 | 68.9 | 0.54 ± 0.0 | 0.50 ± 0.0 |
| | 400 | 7.71 ± 0.3 | 8.25 ± 0.3 | 2.68 ± 0.2 | 2.45 ± 0.1 | 65.8 | 67.9 | 0.50 ± 0.0 | 0.51 ± 0.0 |
| | 500 | 7.16 ± 0.3 | 6.85 ± 0.4 | 2.51 ± 0.2 | 2.40 ± 0.1 | 61.6 | 60.7 | 0.51 ± 0.0 | 0.45 ± 0.0 |
| 72 h | 0 | 12.1 ± 0.5 | 10.8 ± 0.5 | 2.10 ± 0.0 | 2.20 ± 0.1 | 100 | 100 | 0.66 ± 0.0 | 0.67 ± 0.0 |
| | 100 | 10.6 ± 0.3 | 9.70 ± 0.3 | 2.44 ± 0.0 | 2.10 ± 0.1 | 86.3 | 88.6 | 0.58 ± 0.0 | 0.61 ± 0.0 |
| | 200 | 7.69 ± 0.2 | 7.84 ± 0.3 | 2.45 ± 0.1 | 2.20 ± 0.2 | 65.2 | 63.1 | 0.55 ± 0.0 | 0.54 ± 0.0 |
| | 300 | 8.08 ± 0.3 | 7.85 ± 0.4 | 2.56 ± 0.2 | 2.35 ± 0.1 | 68.5 | 67.6 | 0.51 ± 0.0 | 0.48 ± 0.0 |
| | 400 | 6.39 ± 0.3 | 6.65 ± 0.5 | 3.18 ± 0.2 | 2.44 ± 0.1 | 59.1 | 57.1 | 0.47 ± 0.0 | 0.46 ± 0.0 |
| | 500 | 5.67 ± 0.4 | 5.90 ± 0.4 | 3.26 ± 0.1 | 2.57 ± 0.2 | 49.1 | 48.8 | 0.39 ± 0.0 | 0.37 ± 0.0 |

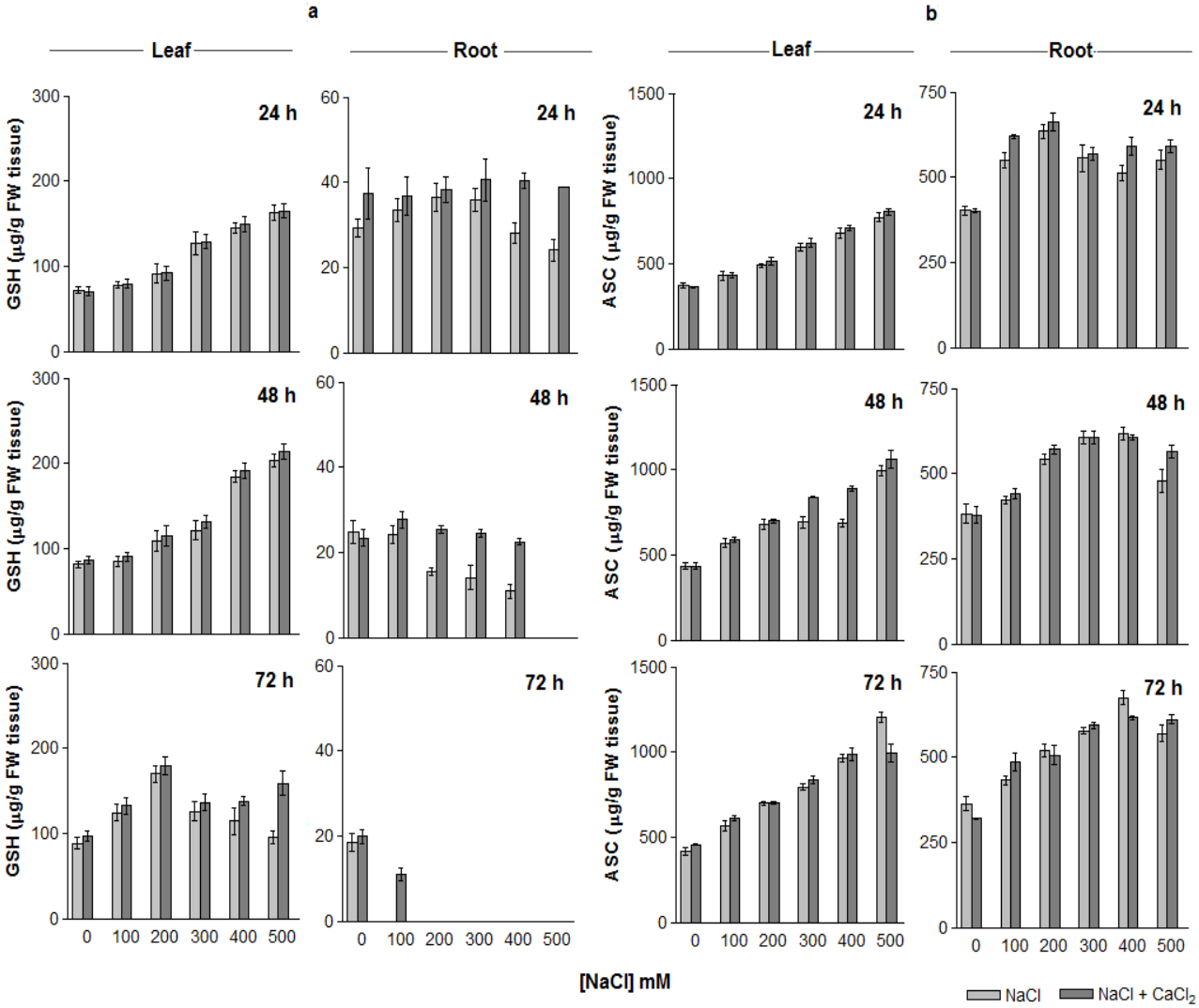


**Fig. 2**: Levels of glutathione, GSH (a); ascorbate, ASC (b) in leaves and roots of Hyacinth bean supplemented with $CaCl_2$. Values are triplicates ± SE ($P \leq 0.05$).

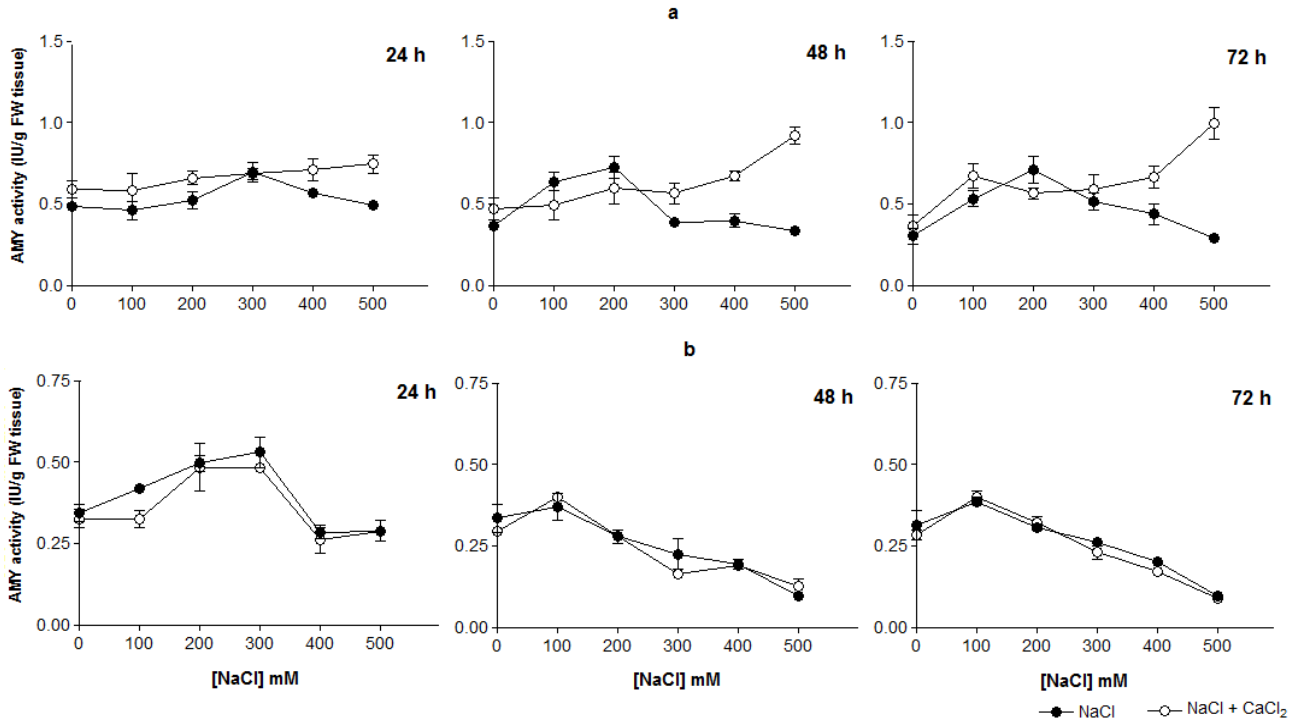


**Fig. 3**: Levels of β-Amylase, AMY activity in leaves (a); roots (b) of Hyacinth bean seedlings after treatment with NaCl (0 to 500 mM) for 24, 48 and 72 h in the presence of exogenous $CaCl_2$. Values are AMY activity ± SE. Each measurement was repeated thrice.

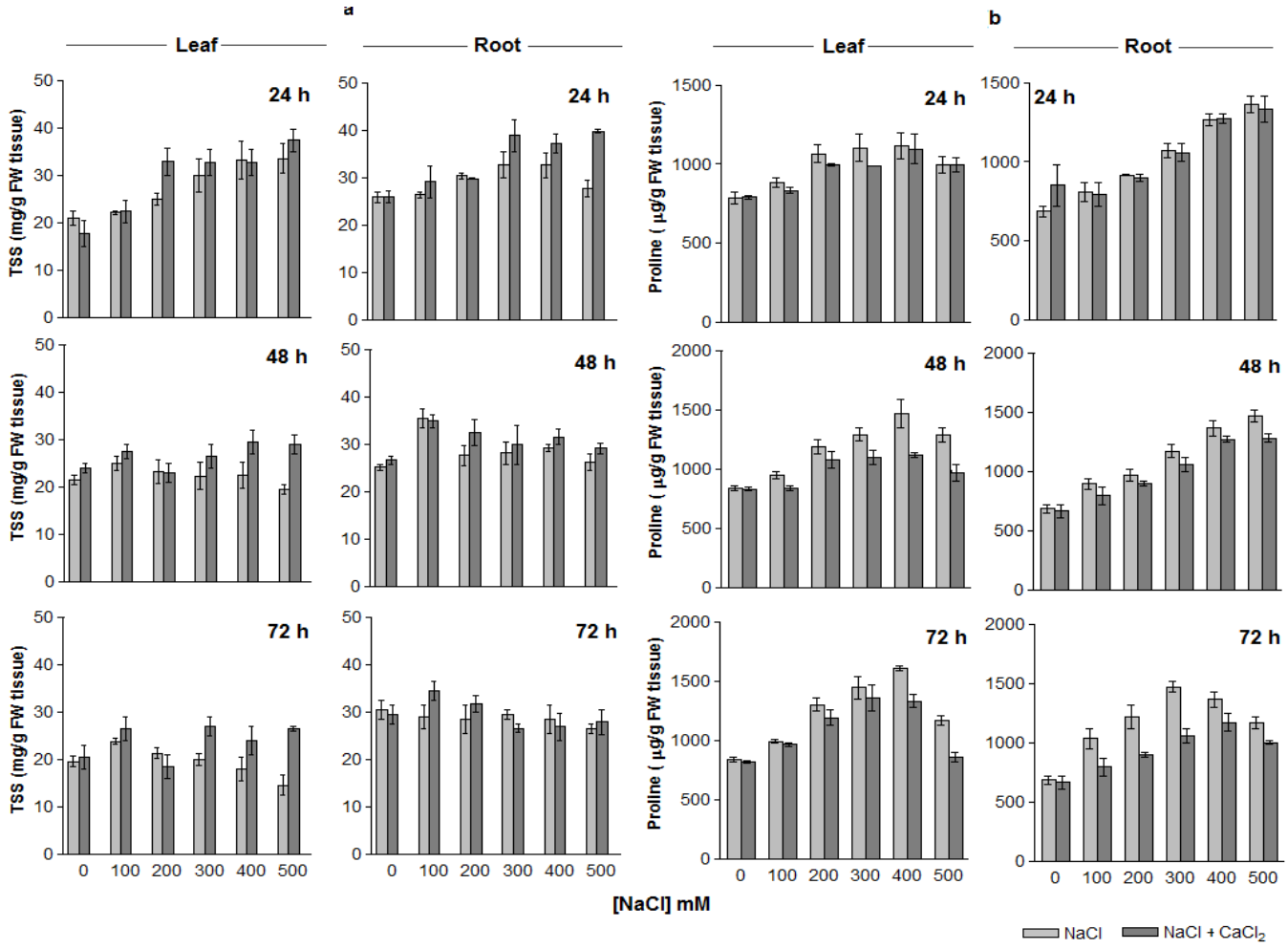


**Fig. 4**: Levels of total soluble sugars, TSS (a); proline (b) in leaves and roots of Hyacinth bean plants. Values are triplicates ± SE ($P \leq 0.05$).

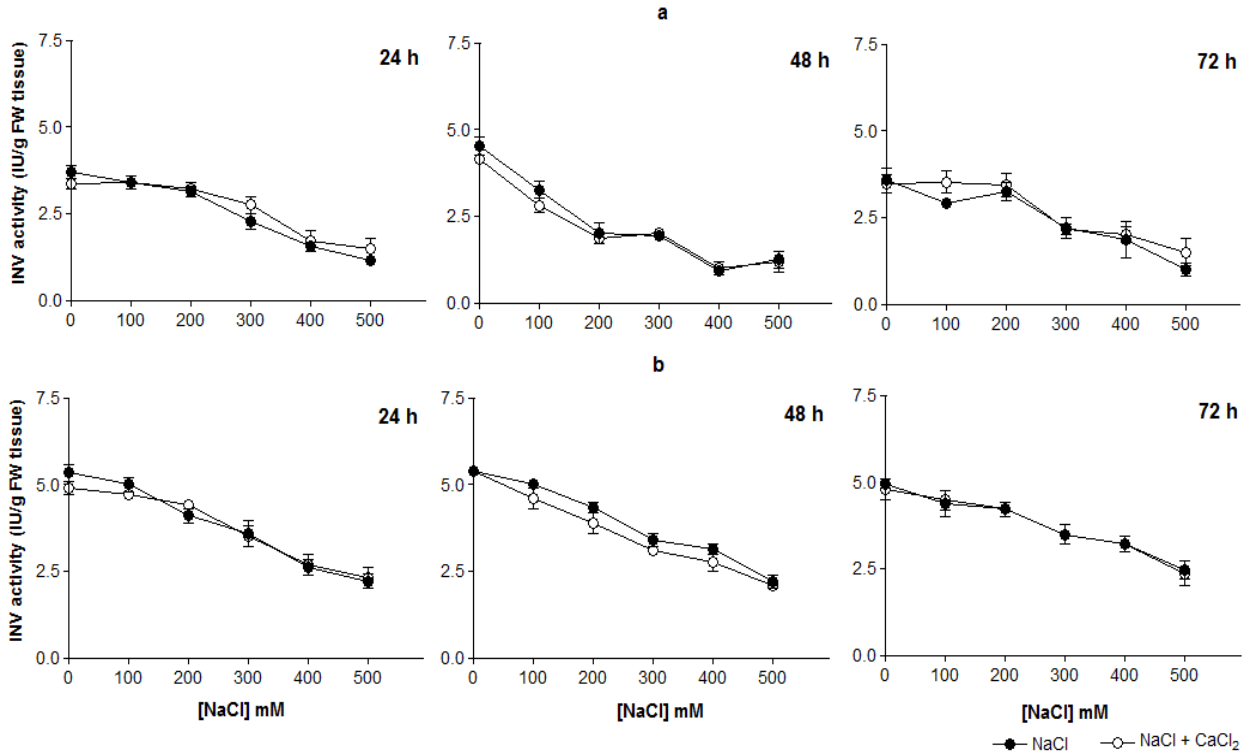


**Fig. 5:** Levels of invertase, INV activity in leaves (a); and roots (b) of Hyacinth bean seedlings after treatment with NaCl (0 to 500 mM) for 24, 48 and 72 h in the presence of exogenous $CaCl_2$. Values are INV activity ± SE. Each measurement was repeated three times.

## 4. CONCLUSION

In conclusion, Hyacinth bean is tolerant to salt up to 300 mM, which is well above the general levels for legumes. The antioxidant system in leaves involves the non-enzymatic components GSH, ASC, and proline. The plant exhibited distinct salt-response mechanisms in leaves and roots, and the root system is less tolerant than the leaf system. Exogenous application of $CaCl_2$ (10 mM) improved the RWC, antioxidants and soluble sugar content and amylase activity; reduced $H_2O_2$ and consequently the MDA levels in both tissues; and helped to ameliorate salinity induced injury to the plant as noticed by morphological and enzymatic studies.

## 5. ACKNOWLEDGEMENT

Myrene R D'souza acknowledges the Council for Scientific and Industrial Research (CSIR), New Delhi, India for SRF.

## 6. REFERENCES

1. FAO, 2006: Extent and causes of salt-affected soils in participating countries. Available at: http://www.fao.org
2. Parida AK, Das AB, Mittra B. and Mohanty P. Salt-stress induced alterations in protein profile and protease activity in the mangrove *Bruguiera parviflora.* Z Naturforsch. 2004; 59 (5-6):408-414.
3. Schoups G, Hopmans JW, Young CA, Vrugt JA, Wallender WW, Tanji KK et al. Sustainability of irrigated agriculture in the San Joaquin Valley, California. Proc Natl Acad Sci. 2005; 102, 15352-15356.
4. Chaves LCG, De Andrade EM, Crisostomo LA, Ness RLL, Lopes JFB. Risk of degradation in irrigated soil at the Distrito de Irrigacao do Perimetro Araras Norte, Ceara, Brazil, Rev Cienc Agron. 2006; 37 (3): 293-299.
5. Rengasamy P. World salinization with emphasis on Australia, J Exp Bot. 2006; 57: 1017-1023.
6. Singh KN, Chatrath R. Salt Tolerance. In: Application of Physiology in Wheat Breeding. CIMMYT Publishers; 2001.
7. Zhu JK. Salt and drought stress signal transduction in plants, Annu Rev Plant Physiol Plant Mol Biol. 2002; 53: 247-273.
8. Hasegawa PM, Bressan RA, Zhu JK, Bohnert HJ. Plant cellular and molecular responses to high salinity, Annu Rev Plant Physiol Plant Mol Biol. 2000; 51: 463-499.
9. Tattini M, Montagni G, Traversi ML. Gas exchange, water relations and osmotic adjustment in *Phillyrea latifolia* grown at various salinity concentrations, Tree Physiol. 2002; 22: 403-412.
10. Cramer GR, Epstein E, Lauchli A. Effect of sodium, potassium and calcium on salt stressed barley. 1. Growth analysis, Physiol Plant. 1990; 80: 83-88.
11. Rengel Z. The role of calcium in salt toxicity, Plant Cell Environ. 1992; 15: 625-632.
12. Watkin ELJ, OW Graham, Glen AR, Identification of tolerance to soil acidity in inoculant strains of *Rhizobium leguminosarum bv. trifolii.* Soil Biology and Biochemistry, 2000; 32 (10): 1393-1403.
13. Cooke A, Cookson A, Earnshaw MJ. The mechanism of action of calcium in the inhibition of high temperature-

induced leakage of betacyanin from beet root discs, New Phytol. 1986; 102: 491-497.
14. Arora R, Palta JP. Plant Physiol. *In Vivo* perturbation of membrane-associated calcium by freeze-thaw stress in onion bulb cells, 1988; 87: 622-628.
15. Bowler C, Fluhr R. The role of calcium and activated oxygens as signals for controlling cross-tolerance, Trends Plant Sci. 2000; 5: 241-246.
16. Mozafari H, KM Kalantari, MS Oliaie, M Torkzadeh, H Salari, S Mirzaei. Role of calcium in increasing tolerance of *Descurainia Sophia* to slat stress, J Agri Soc Sci. 2008; 4: 53-58.
17. Joshi SV, NT Patel, IB Pandey, AN Pandey. Effect of supplemental Ca2+ on NaCl-stressed castor plants (Ricinus communis L.), Acta Bot Croat. 2012; 71(1): 13-29.
18. Tyerman SD, IM Skerrett. Root ion channels and salinity, Sci Hort. 1999; 78: 175-235.
19. Nakamura Y, Tanaka K, Ohta E, Sakata M. Protective effect of external $Ca^{2+}$ on elongation and the intracellular concentration of $K^+$ in intact mung bean roots under high NaCl stress, Plant Cell Physiol. 1990; 31: 815-821.
20. Gallardo M, GM Carmen, A. Matilla. Involvment of calcium in ACC-oxidase activity from *Cicerarietinum* seed embryo nic axes, Phytochemistry 1998; 50: 373-376.
21. Saboury AA, F. Karbassi. Thermodynamic studies on the interaction of calcium ions with alpha-amylase, Thermochim Acta. 2000; 362: 121-129.
22. Nilsen TE, Orcutt MD. Physiology of plants under stress. Wiley; 2000.
23. Allen MM. Simple conditions for growth of unicellular bluegreen algae on plates, J Phycol. 1968; 84: 1-4.
24. Humphries EA. Mineral components and ash analysis, In: Modern Methods of Plant Analysis, Springer-Verlag, Berlin. Gottingen. Heidelberg. 1956.
25. Weimberg R. Changes in growth and water soluble solute concentrations in *Sorghum bicolor* stressed with sodium and potassium, Physiol Plant. 1987; 70: 381-388.
26. Turner NC, Kramer PJ. In: Adaptation of plant to water and high temperature stress. Wiley, New York; 1980.
27. Velikova V, Yordanov I, Edreva A. Oxidative stress and some antioxidant system in acid rain treated bean plants: protective role of exogenous polyamines, Plant Sci. 2000; 151: 59-66.
28. Sadasivam S, Manickam A. Vitamins. In: Sadasivam S, Manickam A, editors. Biochemical methods, 2nd Edition. New Age International (P) Limited, New Delhi. 1997.
29. Beutler E, Duron O, Kelly BM. Improved method for determination of blood glutathione, J Lab Clin Med. 1963; 61: 882.
30. Bates LS, Waldren RP, Teare ID. ) Rapid determination of free proline for water stress studies, Plant Soil. 1973; 39: 205-207.
31. Heath RL, Packer L. Photoperoxidation in isolated chloroplasts. Kinetics and stoichiometry of fatty acid peroxidation, Arch Biochem Biophys. 1968; 125: 189-198.
32. Mackinney G. Absorption of light by chlorophyll solutions, J Biol Chem. 1941; 140: 315-322.
33. Murty KS, Majumder SK. Modifications of technique for determinate, on of chlorophyll stability index in relation to studies of drought resistance in rice Curr Sci.1962;31:470-471.
34. Roe JH. The determination of sugar in blood and spinal fluid with anthrone reagent, J Biol Chem. 1955; 212: 335-343.
35. Lowry OH, Rosebrough NJ, Farr AR, Randoll RJ. Protein measurement with Folin–Phenol reagent, J Biol Chem. 1951; 193: 265-275.
36. Bernfeld P. Amylase α and β, Methods Enzymol. 1955; 1: 149-151.
37. Sridhar R., Ou SH. Extracellular enzymes produced by *Pyricularia oryzae*, Philipp Phytopathol. 1972; 8: 52-56.
38. Miller GL. Use of dinitrosalicylic acid reagent for the determination of sugar, Anal Chem. 1959; 31: 426.
39. Liang W, Wang M, Ai X. The role of calcium in regulating photosynthesis and related physiological indexes of cucumber seedlings under low light intensity and suboptimal temperature stress, Scientia Horticulturae, 2009; 123: 34-38.
40. Posmyk MM, KM Janas. Effects of seed hydropriming in presence of exogenous proline on chilling injury limitation in *Vigna radiata* L. seedlings, Acta Physiol Plant. 2007; 29: 509-517.
41. Cramer GR. Kinetics of maize leaf elongation. II. Response of a Na-excluding cultivar and a Na-including cultivar to varying Na/Ca salinities, J Exp Bot. 1992; 43: 857-864.
42. Jacoby B. Sodium retention in excised bean stem, Physiol Plantarum. 1965; 18: 730-739.
43. Heikal MM, Shaddad MA. Alleviation of osmotic stress on seed germination and seedling growth of cotton, pea and wheat by proline, Phyton (Aust.), 1982; 22: 275-287.
44. Abd El-Samad HM. Response of some plants to the interactive effects of salinity and amino acids, 1987; M.Sc. Thesis, Minia , Egypt.
45. Lynch J, Cramer GR., Läuchli A. Salinity reduces membrane-associated calcium in corn root protoplasts, Plant Physiol. 1987; 83: 390-394.
46. Cramer GR, Läuchli A., Polito VS. Displacement of $Ca^{2+}$ by $Na^+$ from the plasmalemma of root cells. A primary response to salt stress? Plant Physiol. 1985; 79: 207-211.
47. Rubio F, Gassman W, Schroeder JI. Sodium-driven potassium uptake by the plant potassium transporter HKT1 and mutations conferring salt tolerance, Science. 1995; 270: 1660-1663.
48. Gomez MJ, Luyten K, Ramos J. The capacity to transport potassium influences sodium tolerance in *Saccharomyces cerevisiae*, FEMS Microbiol Lett. 1996; 135: 157-160.
49. Kakimoto T. CKI1, a histidine kinase homolog implicated in cytokinin signal transduction, Science. 1996; 274: 982-985.
50. Tsai YC, Hong CY, Liu LF, Kao CH. Relative importance of $Na^+$and $Cl^-$ in NaCl-induced antioxidant systems in roots of rice seedlings, Physiologia Plantarum. 2004; 122: 86-94.
51. Panda SK, Upadhyay RK. Salt stress injury induces oxidative alterations and antioxidative defence in the roots of *Lemna minor*, Biologia Plantarum. 2003; 48: 249–253.
52. Elkahoui S, Hemandez JA, Abdelly C, Ghrir R, Limam F. Effects of salt on lipid peroxidation and antioxidant enzyme activities of *Catharanthus roseus* suspension cells, Plant Sci. 2005; 168: 607-613.
53. Huang Yuan, Zhilong Bie, Zhixiong Liu, Ai Zhen, Weijuan Wang. Protective role of proline against salt stress is partially related to the improvement of water status and peroxidase enzyme activity in cucumber, Soil Science and Plant Nutrition. 2009; 55(5): 698-704.

54. Buettner GR, Jurkiewicz BA. Chemistry and Biochemistry of Ascorbic Acid. In: Handbook of Antioxidants, New York: Dekker, 1996.
55. Padh H. Cellular functions of ascorbic acid, Biochem Cell Biol. 1990; 68: 1166-73.
56. Wingate VPM, Lawton MA, Lamb CJ. Glutathione causes a massive and selective induction of plant defense genes, Plant Physiol. 1988; 31: 205-211.
57. Shaul O. Mironov V, Burssens S, Van Montagu M, Inzé D. Two Arabidopsis cyclin promoters mediate distinctive transcriptional oscillation in synchronized tobacco BY-2 cells; Proc Natl Acad Sci. USA. 1996; 93: 4868-4872.
58. Lappartient AG, Touraine B. Glutathione-mediated regulation of ATP sulfurylase activity, SO42-uptake, and oxidative stress response in intact canola roots, Plant Physiol. 1997; 114: 177-183.
59. Yang SH, Wang LJ, Li SH. Ultraviolet-B irradiation induced freezing tolerance in relation to antioxidant system in winter wheat (*Triticum aestivum* L.) leaves, Environ Exp Bot. 2007; 60: 300-330.
60. Wolfgang D, Schnarrenberger C, Borner TJ, Light-and stress-dependent enhancement of amylolytic activities in white and green barley leaves: β-amylases are stress-induced proteins, Plant Physiol. 1995; 145: 342-348.
61. Sharkey TD, Laporte M, Lu Y, Weise S, Weber AP. Engineering plants for elevated $CO_2$: a relationship between starch degradation and sugar sensing, Plant Biol. 2004; 6: 280-288.
62. Todaka D, Matsushima H, Morohashi Y. Water stress enhances beta-amylase activity in cucumber cotyledons. J Exp Bot. 2000; 51: 739-745.
63. Jaleel CA, Manivannan P, Kishore Kumar A, Sankar B, Panneerselvam R. Calcium chloride effects on salinity induced oxidative stress, proline metabolism and indole alkaloid accumulation in *Catharanthus roseus*, Comptes Rendus Biologies. 2007; 330: 674-683.
64. Jaleel CA., Manivannan P, Lakshmanan GMA, Sridharan R, Panneerselvamn R. NaCl as a physiological modulator of proline metabolism and antioxidant potential in *Phyllanthus amarus*, Comptes Rendus Biologies. 2007; 330: 806-813.
65. Hare PD, WA Cress, Van Staden J. Metabolic implications of stress-induced proline accumulation in plants, Plant Growth Regul. 2003; 39: 41-50.
66. Nayer M, Reza H. Drought induced accumulation of soluble sugars and proline in two maize varieties, World Appl Sci J. 2008; 3(3): 448-453.
67. Schmid VH. Light-harvesting complexes of vascular plants, Cell Mol Life Sci. 2008; 65: 3619-3639.
68. This D, Borries C, Souyris I. Teulat B. QTL study of chlorophyll content as a genetic parameter of drought tolerance in barley, Barley genetics newsletter. 68; 30, 20-23.
69. Parida AK. Das AB. Salt tolerance and salinity effects on plants: a review, Ecotoxicol Environ Safety. 2005; 60: 324-349.
70. Meloni DA, Oliva MA, Martinez CA, Cambraia J. Photosynthesis and activity of superoxide dismutase, peroxidase and gutathione reductase in cotton under salt stress, Environ Exp Bot. 2003; 49: 69-79.
71. Yasar F. Effects of salt stress on ion and lipid peroxidation content in Green beans genotypes, Asian J Chem. 2007; 19: 1165-1169.
72. Singh G., Jain S. Effect of some growth regulators on certain biochemical parameters during seed development in chickpea under salinity, Indian J Plant Physiol. 1982; 20: 167-179.
73. Anderson JM. Photoregulation of the composition, function, and structure of thylakoid membranes, Annu Rev Plant Physiol 1986;. 37: 93-136.
74. Djanaguiraman M, Ramadass R. Effect of salinity on chlorophyll content of rice genotypes, Agric Sci Digest. 2004; 24(3): 178-181.
75. Ashraf M, Ashfaq A, McNeilly T. Photosynthetica. Growth and Photosynthetic characteristics in Pearl Millet under water stress and different potassium supply, 2001; 39, 389-394.
76. Telfer A, Too much light? How β-carotene protects the photosystem II reaction centre, Photochem Photobiol Sci. 2005; 4: 950-956.
77. Farooq M, Wahid A., Kobayashi N, Fujita D, Basra, SMA. Plant drought stress: effects, mechanisms and management, Agron Sustain Dev. 2009; 29: 185-212.
78. Sharma PK, Hall DO. Interaction of salt stress and photoinhibition o photosynthesis in barley and sorghum, J Plant Physiol. 1991; 138: 614-619.
79. Shetty K. Role of proline-linked pentose phosphate pathway in biosynthesis of plant phenolics for functional food and environmental applications: a review, Process Biochem. 2004; 39: 789-803.
80. Girija C., Smith BN, Swamy PM. Interactive effects of sodium chloride and calcium chloride on the accumulation of proline and glycinebetaine in peanut (*Arachis hypogaea* L.), Environm Exp Bot. 2002; 47: 1-10.
81. Ermak G., Kelvin DJ. Calcium and oxidative stress: from cell signaling to cell death, Mol Immunol. 2000; 38: 713-721.



**How to cite this article:**

Myrene R. D'souza and Devaraj V. R., Role of calcium in increasing tolerance of Hyacinth bean to salinity. J App Biol Biotech, 2013; 1 (03): 011-020.