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## Calcium availability regulates antioxidant system, physio-biochemical activities and alleviates salinity stress mediated oxidative damage in soybean seedlings

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### Summary

Salinity is considered as one of the devastating abiotic stress factors and global climate change has further worsened the situation. Present experiments were aimed to evaluate the role of calcium (Ca) availability on growth and salinity tolerance mechanisms in soybean. Seedlings were grown with (2 mM Ca) and without Ca supplementation and modulation in key physiological and biochemical parameters were studied. Salinity (100 mM NaCl) stress resulted in growth reduction in terms of height and biomass accumulation, which was more pronounced in Ca-deficient plants. Relative to control (Ca deficient) and NaCl stressed plants, Ca supplemented seedlings exhibited higher relative water content, pigment synthesis and the photosynthetic efficiency. Ca availability affected the synthesis of proline, glycine betaine and soluble sugars under normal and saline growth conditions. Optimal Ca supplementation up-regulated the activities of antioxidant enzymes assayed and the contents of non-enzymatic antioxidants (ascorbate, glutathione, and tocopherol) thereby reflecting in amelioration of NaCl induced oxidative damage. Moreover, increased accumulation of phenols due to Ca supplementation and the amelioration of NaCl mediated decline in nitrate reductase activity was observed. More importantly, Ca availability reduced the accumulation of Na under control and NaCl stressed conditions restricting the damaging effects on metabolism. Availability of optimal Ca potentially regulates the salinity tolerance mechanisms in soybean by maintaining osmoregulation and antioxidant metabolism.

**Keywords:** Calcium, Antioxidant system, Osmolytes, Lipid peroxidation, *Glycine max* L.

### Introduction

Increasing salinity in soils has become one of the serious threats to distribution, survival, and productivity of major crop plants (ASHRAF and HARRIS, 2004). Increased salinity hampers key growth and metabolic processes including photosynthesis, ion transport their assimilation and distribution, enzyme activity and osmolyte metabolism (IQBAL et al. 2015). Salinity interferes with carbon and nitrogen metabolism in plants leading to alterations in the physiological processes responsible for growth and developmental regulation (AHANGER and AGARWAL, 2017; ELKELISH et al., 2019). Presence of high concentration of toxic ions like sodium in the soil hampers the uptake of important nutrients including nitrogen, sulfur, calcium, and potassium (REDA et al., 2011). Salinity stress imposes hyper-osmotic and hyper-ionic stress in plants leading to membrane disorganization and restriction of functioning in chloroplast and mitochondria. Higher

salinity declines the soil osmotic potential thereby prevent the uptake of water into the plant ultimately due to occurrence of physiological drought often reflected as reduced growth and development (OZDEN et al., 2009). It has been reported that salinity stress increases the generation of toxic reactive oxygen species (ROS) and plants have an antioxidant system comprised of enzymatic and non-enzymatic components to neutralize ROS and safeguard the cellular functioning (NOCTOR and FOYER, 2016).

Increased generation of ROS due to stress affects the macromolecules like proteins, nucleic acids etc. (AHANGER and AGARWAL, 2017) rendering plant cells less efficient. It has been reported that salinity generated ROS reduce the rate of photosynthesis, metabolism of nitrogen and carbohydrates often reflected in reduced growth rate and yield (AHANGER and AGARWAL, 2017; SOLIMAN et al., 2018). More importantly, salinity influences the expression of proteins regulating the structural and functional integrity of organelles like chloroplast and mitochondria (OMIDBAKHSHFARD et al., 2012). Therefore, for maximally averting the negative effects of salinity stress key mechanisms are initiated to reduce the impact on the growth and productivity of the plant. Salt exclusion through channel proteins, up-regulation of ROS scavenging system and accumulation of osmolytes are included among the basic mechanisms leading to enhanced protection to structure and function of cellular organelles by regulating ROS and stress signaling (SUO et al., 2017). Therefore, strengthening the tolerance mechanisms and understanding their implications on the overall growth performance is requisite for achieving sustainable productivity.

Like other beneficial nutrients, interaction between available calcium (Ca) with the salinity plays an important role in initiating and strengthening of the tolerance mechanism. Salinity reduces uptake of Ca inducing oxidative damage through greater accumulation of ROS. Ca deficiency limits root growth, induces leaf necrosis and curling, affects cellular signaling and can result in blossom end rot, bitter pit and fruit cracking, affect cellular signaling and sensing of other mineral elements like potassium (WANG et al., 2018). Once Na<sup>+</sup> is taken up in excess, leakage of K<sup>+</sup> and Ca<sup>2+</sup> follows resulting in imbalance of the cellular ion homeostasis ultimately causing oxidative stress, growth retardation and programmed cell death (CABOT et al., 2014). The role of optimal Ca availability in growth regulation has not been worked so far. We hypothesized that availability of optimal Ca can ameliorate the deleterious effects of salinity stress by strengthening the key tolerance mechanisms. Therefore, experiments were conducted using one of the important legume crop, soybean, to investigate the role of Ca availability in salinity stress mitigation by evaluating its involvement in the (a) regulation of the antioxidant system and osmolytes, (b) secondary metabolite accumulation and the (c) mineral nutrition.

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## Materials and methods

### Experimental design and treatment

A greenhouse experiment was conducted at the Department of Botany of the Faculty of Science, Suez Canal University, Ismailia, Egypt, during November and December 2018. Soybean (*Glycine max* L.) seeds were sterilized with 3% sodium hypochlorite and sown in earthen pots filled with an equal quantity of reconstituted soil containing peat, compost, and sand in the ratio of 3:1:1. Concentration of available N, P, K and Ca in soil substrate was 53.21, 20.03, 56.28 and 17.33 mg kg<sup>-1</sup> soil respectively. Pots were wetted by applying 200 mL of full-strength nutrient solution. Soon after the germination number of seedlings per pot was maintained five. Pots were grouped into two categories where one group was irrigated with nutrient solution (100 mL per pot) with Ca (in the form of 2 mM Ca (NO<sub>3</sub>)<sub>2</sub>) and another group without Ca. 100 mM NaCl was supplemented in the form of modified Hoagland solution to induce salinity stress. The composition of nutrient solution used was 3 mM KNO<sub>3</sub>, 2 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 2 mM MgSO<sub>4</sub>, 1 mM NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, 50 μM KCl, 25 μM H<sub>3</sub>BO<sub>3</sub>, 2 μM MnCl<sub>2</sub>, 20 μM ZnSO<sub>4</sub>, 0.5 μM CuSO<sub>4</sub>, 0.5 μM (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>, and 20 μM Na<sub>2</sub>Fe-EDTA (PER et al., 2016). Therefore, we have following set of treatments:

- Control (Hoagland solution without Ca).
- Ca (full strength Hoagland solution).
- Salt stressed (Hoagland solution without Ca + 100 NaCl).
- Salt stress + Ca (full strength Hoagland solution + 100 NaCl).

Plants were irrigated on every alternate day. Thirty days old plants were harvested and analyzed for photosynthetic attributes, osmoregulatory components, oxidative stress markers, and antioxidants. During the entire experimental time, period pots were kept in a greenhouse and arranged in a complete randomized block design with five replicates for each treatment.

### Measurement of photosynthetic pigments, stomatal conductance, and photosynthetic efficiency

For extraction of chlorophyll and carotenoids, fresh leaf tissue was homogenized in pestle and mortar using 80% acetone (ARNON, 1949). The homogenate was centrifuged, and an optical density of supernatant was taken at 480, 645 and 663 nm against 80% acetone. For measurement of photosynthetic efficiency and stomatal conductance, fully expanded leaf was analyzed using the infrared gas analyzer (CID-340, Photosynthesis System, Bio-Science, USA).

### Determination of leaf water content, soluble sugars, proline, and glycine betaine content

Relative water content (RWC) of leaves was determined by punching an equal number of leaf discs from each treatment with a sharp cork borer, and their fresh weight (FW) was taken. Thereafter the same leaf discs were floated in petri dishes containing distilled water for 1 hr for measuring the turgid weight (TW). The same discs were oven dried at 80 °C for 24 hrs for the dry weight (DW) measurement (SMART and BINGHAM, 1974). RWC was calculated by the following formula:

$$\text{RWC} = \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \times 100$$

A method described by BATES et al. (1973) was used to estimate the proline content. Dry (500 mg) leaf sample was homogenized in 3% sulphosalicylic acid and subjected to centrifugation for 20 minutes at 3000 g. The supernatant (2 mL) was mixed with 2 mL glacial acetic acid and 2 mL ninhydrin reagent at 100 °C in a water bath for 1 hour. After that samples were cooled on an ice bath and proline was separated using toluene. Absorbance was read spectrophotometrically at 520 nm. Calculation was done from standard curve of proline.

For measurement of soluble sugar, content oven dried samples were macerated in 80% ethanol and after centrifugation supernatant was reacted with anthrone reagent following method of SHIELDS and BURNETT (1960). Calculation was done from standard curve of glucose.

For the determination of glycine betaine (GB) method of GRIEVE and GRATTAN (1983) was followed. Dried plant samples were extracted in distilled water and filtered. The filtrate was diluted by H<sub>2</sub>SO<sub>4</sub> (2 N) and cold KI-I<sub>2</sub> reagent was added to an appropriate volume of the aliquot. Samples were centrifuged at 10,000 g for 15 minutes. The supernatant was aspirated, and per-iodide crystals were dissolved in 1,2-dichloroethane and after two hours absorbance was taken at 365 nm. The calculation was done using a standard of glycine betaine.

### Lipid peroxidation and hydrogen peroxide estimation

For the measurement of lipid peroxidation, the formation of malonaldehyde (MDA) content was estimated. Fresh leaves were homogenized in 1% trichloroacetic acid (TCA) and the homogenate was centrifuged at 10,000 g for 5 min. Thereafter, 1.0 mL supernatant was mixed with 4 mL 0.5% thiobarbituric acid and mixture was boiled for thirty minutes at 95 °C. Tubes were cooled on an ice bath and centrifuged again for 5 min at 5000 g for clarification. The optical density of supernatant was measured at 532 and 600 nm (HEATH and PACKER, 1968).

Hydrogen peroxide was estimated by macerating 0.5 gm fresh leaf tissue in 5 mL of 0.1% TCA and the homogenate was centrifuged for 15 minutes at 12,000 g. Supernatant (0.5 mL) was mixed with 0.5 mL potassium phosphate buffer (pH 7.0) and potassium iodide (1 mL). Tubes were thoroughly shaken by vortexing and the absorbance was taken at 390 nm (VELIKOVA et al., 2000).

### Determination of nitrate reductase

Fresh 500 mg tissue was extracted in ice-cold mortar using 100 mM potassium phosphate buffer (pH 7.5) containing 5 mM cysteine, 1 mM EDTA and 0.5% PVP. The homogenate was centrifuged at 15,000 g for 20 min and the supernatant was used for the determination of NR activity. The reaction mixture consisted 100 mM potassium phosphate buffer (pH 7.5), 7 mM KNO<sub>3</sub>, 10 mM MgCl<sub>2</sub>, 0.14 mM NADH and the enzyme extract. The reaction was initiated by addition of NADH and after incubation for 30 minutes at 27 °C, the reaction was terminated by addition of 100 μL zinc acetate (0.5M) followed by centrifugation for 10 minutes at 3000 g and subsequent addition of 1% sulfanilamide and 0.01% naphthalenedi-amine-dihydrochloride (NED). After 20 minutes the absorbance was read at 540 nm (DEBOUBA et al., 2006).

### Assay of antioxidant enzymes

Antioxidant enzymes were extracted by homogenizing 5.0 gm of fresh leaf tissue in pre-chilled pestle and mortar in phosphate buffer (50 mM, pH 7.0) supplemented with 1% polyvinyl pyrrolidone and 1 mM EDTA. The homogenate centrifuged at 15,000 g for 20 min at 4 °C and supernatant was used as an enzyme source. Protein content in the supernatant was determined by LOWRY et al. (1951) method. The assay of superoxide dismutase (SOD, EC 1.15.1.1) was carried by measuring the ability of enzyme extract to inhibit the photochemical reduction of nitroblue tetrazolium chloride (NBT) at 560 nm (BEYER and FRIDOVICH, 1987). The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.5), 0.1 μM EDTA, L-methionine (13 mM), 75 μM of each NBT and riboflavin, and 100 μL enzyme extract in a final volume of 1.5 mL. Samples were shaken and illuminated for 15 min and the reaction was terminated by switching off the light. The absorbance of the enzyme containing illuminated samples was recorded against their respective non-illuminated blank at 560 nm.

The amount of enzyme causing 50% inhibition in NBT photoreduction was considered as one unit of SOD and activity was expressed as EU  $\text{mg}^{-1}$  protein.

For measuring the activity of ascorbate peroxidase (APX, EC 1.11.1.11) decrease in absorbance was monitored at 290 nm for 3 min in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), ascorbic acid (0.5 mM), hydrogen peroxide (0.1 mM) and 100  $\mu\text{L}$  of enzyme extract in final volume of 1 mL (NAKANO and ASADA, 1981). The extinction coefficient of  $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$  was used for calculation.

For the assay of glutathione reductase (GR; EC 1.6.4.2) method of FOYER and HALLIWELL (1976) was adopted. Briefly, assay mixture (1.0 mL) contained sodium phosphate buffer (50 mM, pH 7.8), 0.12 mM nicotinamide adenine dinucleotide phosphate (NADPH), 0.5 mM oxidized glutathione (GSSG) and 100  $\mu\text{L}$  enzyme extract. Change in absorbance at 340 nm was followed for 2 min. Activity was expressed as 1 mol NADPH oxidized  $\text{min}^{-1}$  (units  $\text{mg}^{-1}$  protein) and extinction coefficient for of  $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$  was used for calculation.

#### Determination of ascorbate, tocopherol and reduced glutathione

For the determination of ascorbate (AsA) content 400 mg fresh plant material of each treatment was extracted in 5 mL of 6% TCA followed by centrifugation at 5000 g for 10 min. To 1 mL supernatant was added 2% dinitrophenylhydrazine (prepared in 9 M  $\text{H}_2\text{SO}_4$ ) and 10% thiourea. The mixture was boiled in a water bath for 15 min and cooled followed by the addition of 5 mL of cooled 80%  $\text{H}_2\text{SO}_4$ . Thereafter the optical density was measured at 530 nm (MUKHERJEE and CHOUDHURI, 1983) and calculation was done from a standard curve of ascorbate.

The method described by (BAKER et al., 1980) was followed for the estimation of tocopherol. 100 mg fresh leaf material was extracted with 5 mL of ethanol and petroleum ether (1.6:2) and an extract was centrifuged at 12,000 g for 10 minutes. 1.0 mL supernatant was mixed with 0.2 mL of 2, 2-dipyridyl (2%, prepared in ethanol) and left for 5 min in dark for color (red) development. Thereafter 4 mL of distilled water was added and the absorbance was measured at 520 nm.

Reduced glutathione (GSH) was estimated by extracting fresh 100 mg leaf tissue in phosphate buffer (pH 8.0) and an extract was centrifuged for 15 minutes at 3000 g. Thereafter 500  $\mu\text{L}$  of supernatant was reacted with 5, 5-dithiobis-2-nitrobenzoic acid and allowed to stand for 10 minutes. Optical density was measured at 412 nm (ELLMAN, 1959) and the concentration of GSH was determined from a standard graph of GSH.

#### Estimation of total phenols

Dry (500 mg) plant samples were extracted in 80% ethanol and an extract was centrifuged at 10,000 g for 10 minutes. The supernatant was mixed with Folin and Ciocalteu's phenol reagent in alkaline medium and optical density of the mixture was recorded at 750 nm (SLINKARD and SINGLETON, 1977). The computation was done from the standard curve of catechin.

#### Estimation of ions

For estimation of Na and Ca, one gm oven dried samples were acid digested using  $\text{H}_2\text{SO}_4$  and  $\text{HClO}_4$ . Digested samples were diluted to 100 mL using distilled water and were read on flame photometer.

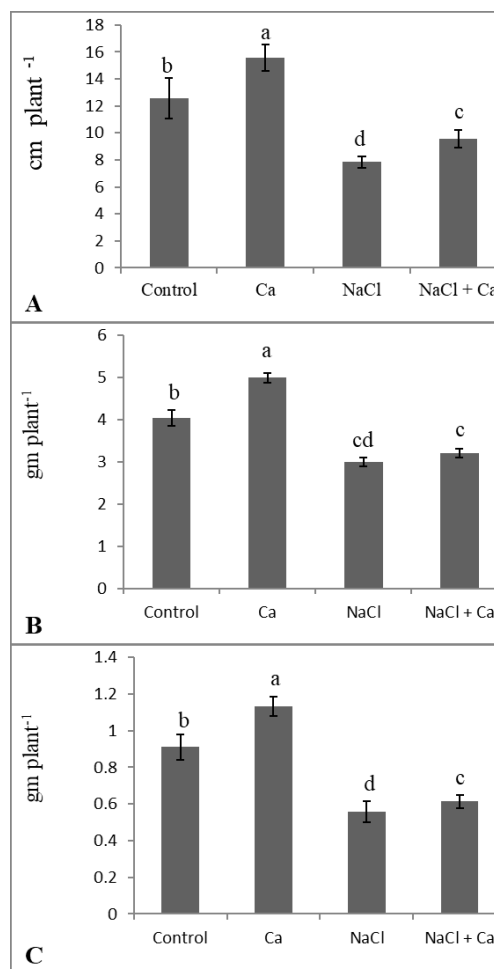
#### Statistical analysis

Data are mean ( $\pm$ SE) of three replicates and Duncan's Multiple Range Test was performed using One Way ANOVA for determining the least significant difference (LSD) at  $p < 0.05$ .

## Results

### Ca supplementation protects plant growth and biomass production under salinity stress

Results showing the impact of Ca availability on the height and biomass accumulation under salinity and normal conditions are presented in Fig. 1. Relative to control, Ca improved height, fresh and dry weight by 19.30, 19.19 and 19.58% respectively. Salinity (100 mM NaCl) reduced the height (37.67%), fresh (25.79%), and dry (38.81) biomass accumulation over the control plants. Application of Ca significantly reduced the negative effects of NaCl and ameliorated the decline by 17.90, 12.78 and 9.23% over the NaCl stressed counterparts (Fig. 1).



**Fig. 1:** Effect of salinity (100 mM NaCl) on (A) shoot length, (B) fresh and (C) dry weight of soybean (*Glycine max* L.) with and without calcium supplementation. Data is mean ( $\pm$ SE) of three replicates and bars with different letters denote significant difference at  $P \leq 0.05$

### Ca supplementation improves photosynthetic efficiency and the pigment synthesis

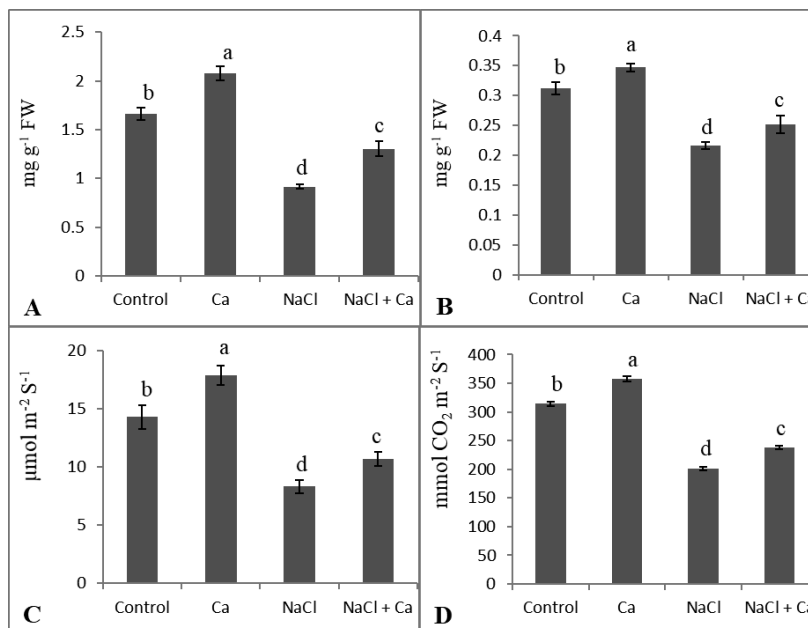
Supplementation of Ca resulted in significant enhancement in the pigment synthesis (chlorophylls and carotenoids) and also ameliorated the NaCl induced decline. Relative to control, total chlorophylls, carotenoids, photosynthetic rate, and stomatal conductance increased by 19.97, 17.25, 20.15 and 14.44% in Ca treated seedlings. However, NaCl stress reduced chlorophylls (45.18%), carotenoids (30.72%), photosynthetic rate (41.88%) and stomatal conductance (35.98%) over the control plants. The reduction was mitigated by supplementation of Ca with an amelioration of 29.64% in total chlorophylls,

14.10% in carotenoids, 22.33% photosynthetic rate and 15.54% in stomatal conductance over the NaCl stressed plants (Fig. 2A-D).

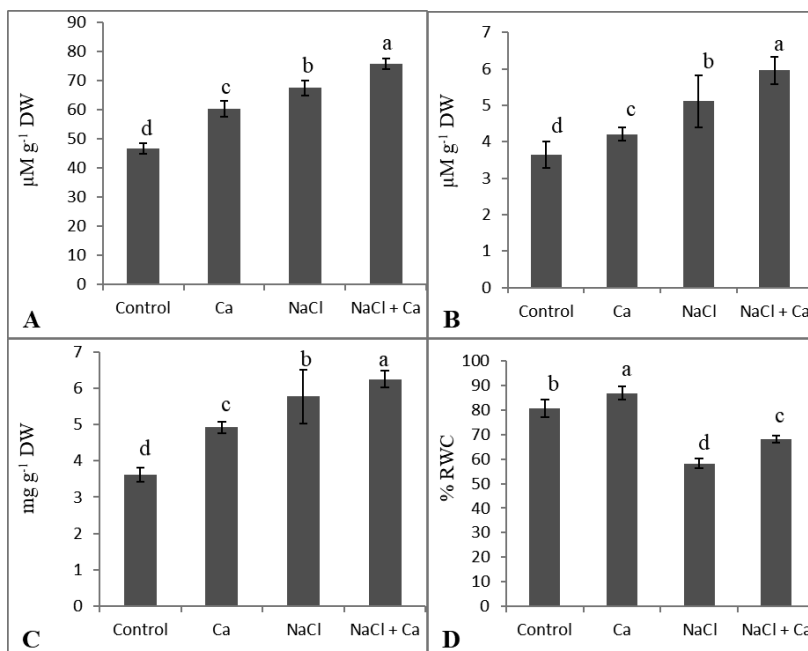
### Ca availability improves RWC, and proline, glycine betaine and sugar accumulation under salinity stress

In normally grown seedlings, Ca increased the accumulation of proline (Pro), glycine betaine (GB) and soluble sugar content significantly over the Ca deficient control plants. An increment of 22.63% in Pro, 14.28% in GB and 26.53% sugar content was observed over the

control plants. Percent increase in Pro, GB, and sugars due to NaCl stress was 31.01, 28.96, and 37.43% respectively over the control. The maximal increase in Pro (38.39%), GB (39.49%) and sugar (42.14%) content was observed in NaCl + Ca treated plants over the control counterparts (Fig. 3A-C). A reduction of 27.85% was observed in RWC over the control seedlings, however, Ca was effective in improving and also mitigating the negative effects of NaCl on RWC. An increase of 7.16% in RWC was observed due to Ca over control and caused an amelioration of 14.57% in seedlings treated with NaCl + Ca over the NaCl stressed plants (Fig. 3D).



**Fig. 2:** Effect of salinity stress (100 mM NaCl) on (A) total chlorophylls, (B) carotenoids, (C) photosynthetic rate and (D) stomatal conductance in soybean (*Glycine max* L.) with and without calcium supplementation. Data is mean ( $\pm$ SE) of three replicates and bars with different letters denote significant difference at  $P \leq 0.05$



**Fig. 3:** Effect of salinity stress (100 mM NaCl) on content of (A) proline, (B) glycine betaine, (C) soluble sugars and (D) relative water content (RWC) in soybean (*Glycine max* L.) with and without calcium supplementation. Data is mean ( $\pm$ SE) of three replicates and bars with different letters denote significant difference at  $P \leq 0.05$

### Antioxidant system was up-regulated due to Ca supplementation

Supplementation of Ca resulted in a significant increase in the activities of SOD, APX, and GR, and the contents of AsA, GSH, and tocopherol (Fig. 5 and 6). Relative to control, an increase of 21.37% in SOD, 19.73% in APX and 9.46% in GR were observed due to supplementation of Ca. NaCl stress increased the activities of SOD (36.54%), APX (40.00%) and GR (33.40%) over the control, however application of Ca further enhanced the activities. Relative to control, an increase of 44.98, 45.37 and 39.26% in SOD, APX and GR were observed due to an application of Ca to NaCl stressed plants (Fig. 5A-C). Contents of AsA, GSH, and tocopherol increased by 8.89, 9.91 and 21.97% in Ca treated plants over the Ca deficient controls (Fig. 6A-C). The maximal increase in GSH (30.06%) and tocopherol (35.36%) was observed in seedlings treated with NaCl + Ca over the control. Salinity reduced AsA (15.57%) while as increased GSH (26.55%) and tocopherol (14.48%) over the control. Reduction in AsA was ameliorated by application of Ca and an amelioration of 4.67% in AsA content was observed in NaCl + Ca treated seedlings over the NaCl stressed plants (Fig. 6A).

### Ca reduces oxidative damage by reducing the formation of hydrogen peroxide and lipid peroxidation

It was observed that Ca supplementation reduced the generation of  $H_2O_2$  and lipid peroxidation significantly over the control as well as NaCl stressed plants (Fig. 4 A and B). Accumulation of  $H_2O_2$  increased by 37.98% in NaCl stressed plants causing 25.15% increase in lipid peroxidation over the control plants. Supplementation of optimal Ca reduced the generation of  $H_2O_2$  by 23.29% over the control leading to a declined lipid peroxidation by 13.38% in them. In comparison to NaCl stressed plants amelioration of 17.56% in  $H_2O_2$  and 11.59% in lipid peroxidation was observed in seedlings treated with NaCl + Ca (Fig. 4 A and B).

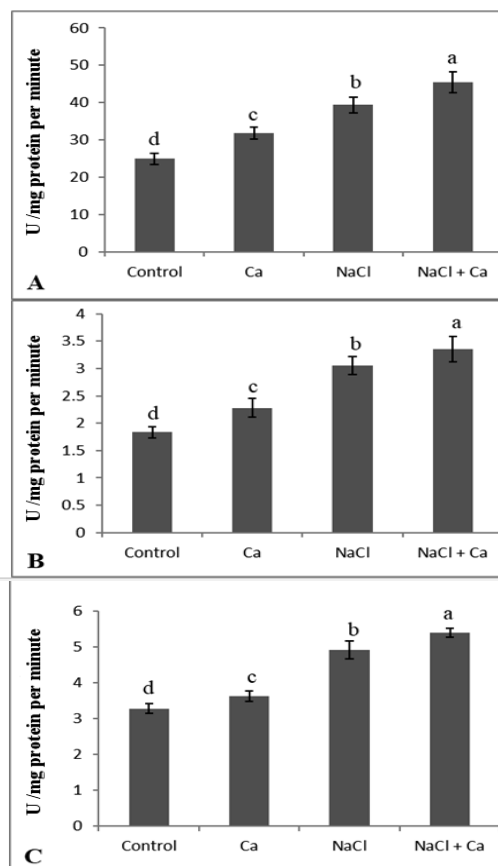
### Ca improves the synthesis of total phenols and enhances nitrate reductase activity

Ca supplemented seedlings exhibited an apparent increase in the accumulation of total phenols over the Ca deficient normal and salinity stressed plants. Relative to control, Ca supplemented plants showed an increase of 22.39% in total phenol contents which was reduced by 18.33 % due to NaCl stress (Fig. 7).

Relative to control, treatment of 100 mM NaCl reduced the activity of nitrate reductase (NR) by 43.25%. However, an amelioration of 11.96% was observed in NaCl + Ca over the NaCl stressed plants. Under normal growth conditions, NR activity increased (11.89%) significantly due to Ca availability (Fig. 8).

### Ca availability reduces Na uptake and reduces the Na/K ratio

Supplementation of Ca reduced the uptake of Na to upper parts (leaf) significantly (Tab. 1). Relative to Ca deficient control, Ca decreased

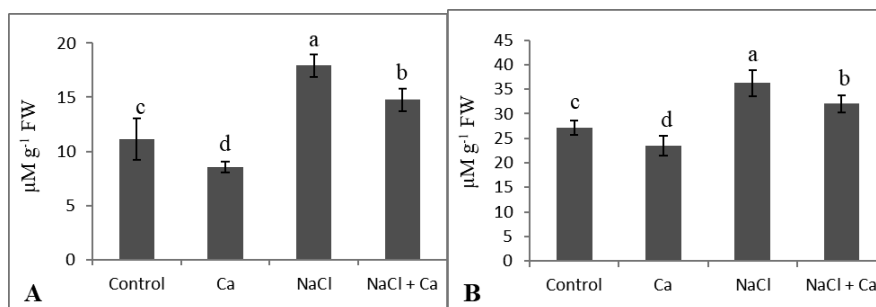


**Fig. 5:** Effect of salinity stress (100 mM NaCl) on activity of (A) superoxide dismutase (B) ascorbate peroxidase and (C) glutathione reductase in soybean (*Glycine max* L.) with and without calcium supplementation. Data is mean ( $\pm$ SE) of three replicates and bars with different letters denote significant difference at  $P \leq 0.05$

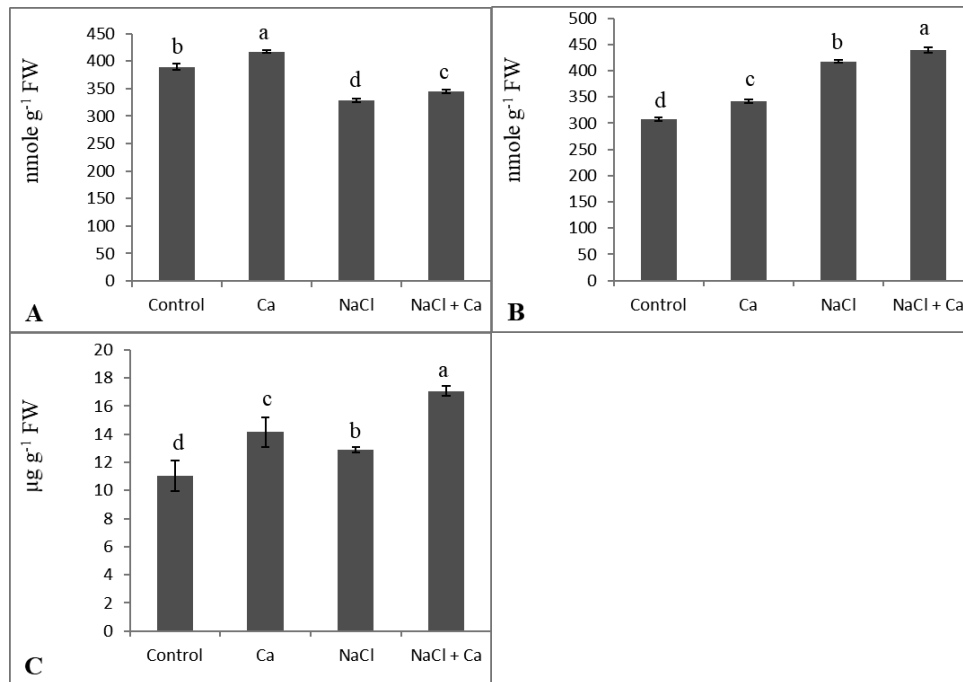
by 35.13% in leaf and 31.75% in root due to NaCl stress, however, accumulated Na in leaf (68.54%) and root (65.98%) more than the control. Relative to control, Ca supplemented seedlings exhibited an increase in the leaf Ca (24.38%), while a decrease in Na content of leaf (28.53%) and root (13.15%). However, in NaCl stressed plants Ca application reduced the Na accumulation by 42.26% and 32.65% in leaf and root tissues (Tab. 1).

## Discussion

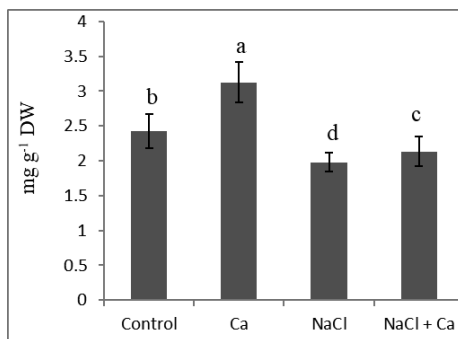
Increased soil salinity is considered a critical threat to optimal crop productivity. Such problems have arisen mainly due to the introduction of agricultural malpractices like the use of salt-rich and polluted



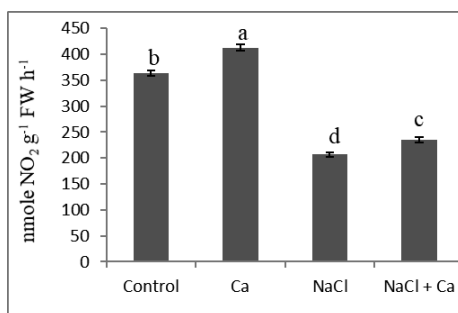
**Fig. 4:** Effect of salinity stress (100 mM NaCl) on (A) hydrogen peroxide and (B) lipid peroxidation (MDA) in soybean (*Glycine max* L.) with and without calcium supplementation. Data is mean ( $\pm$ SE) of three replicates and bars with different letters denote significant difference at  $P \leq 0.05$



**Fig. 6:** Effect of salinity stress (100 mM NaCl) on content of (A) ascorbic acid, (B) reduced glutathione and (C) tocopherol in soybean (*Glycine max* L.) with and without calcium supplementation. Data is mean ( $\pm$ SE) of three replicates and bars with different letters denote significant difference at  $P \leq 0.05$



**Fig. 7:** Effect of salinity stress (100 mM NaCl) on content of total phenols in soybean (*Glycine max* L.) with and without calcium supplementation. Data is mean ( $\pm$ SE) of three replicates and bars with different letters denote significant difference at  $P \leq 0.05$



**Fig. 8:** Effect of salinity stress (100 mM NaCl) on content the activity of nitrate reductase in soybean (*Glycine max* L.) with and without calcium supplementation. Data is mean ( $\pm$ SE) of three replicates and bars with different letters denote significant difference at  $P \leq 0.05$

water for irrigations, which has resulted in continuous accumulation of salts to toxic levels making the growth and development of growing crops difficult. For ensuring better crop productivity under such growth conditions, efficient and robust management practices need to be introduced. In this context, we investigated the potential of soil Ca availability in mitigating the adverse effects of salinity.

The indigenous concentration of Ca in nutrient solution proved effective in ameliorating the NaCl induced growth decline. NaCl reduces the cellular growth and division by reducing the activity of cyclin-dependent kinase and *CYCBI;2* promoters resulting in the production of smaller meristems (WEST, 2004). Salinity stress-mediated decline in meristem is due to the reduced cellular water content (MAHMOODZADEH et al., 2007). In the present study, Ca application effectively regulated the growth of soybean by improving biomass production and growth under salinity stress. Ca is important for cell cycle progression under stress and is an important secondary messenger. The role of Ca<sup>2+</sup> mediated signaling in cell cycle progression has been established (MACHACA, 2010) availability of optimal Ca concentration in the present study may have prevented cell cycle arrest besides regulating the cell proliferation (KAHL and MEANS, 2003). As a signaling molecule Ca ion acts as a convergence point of many signaling pathways thereby helping plant cells to reprogram their cellular set up in response to stress signal (TUTEJA and MAHAJAN, 2007). Ca helps in stress signal perception by membrane receptors, secondary messenger generation, protein phosphorylation and dephosphorylation targeting transcription factors resulting in regulation of gene expression ultimately imparting tolerance to stress (TUTEJA and MAHAJAN, 2007).

Presence of optimal Ca in solution resulted in significant improvement in the synthesis of chlorophylls and carotenoids resulting in greater photosynthetic efficiency and stomatal conductance. Earlier XU et al. (2013) have also demonstrated that the exogenous application of CaCl<sub>2</sub> ameliorated the negative effects of drought stress on growth by improving the chlorophyll pigment synthesis and the chlorophyll fluorescence and photosynthetic rate. Ca deficiency intensified the reduction in photosynthetic pigment and performance

**Tab. 1:** Effect of salinity stress (100 mM NaCl) on the uptake of sodium and calcium ( $\text{mg g}^{-1}$  DW) in soybean (*Glycine max* L.) with and without calcium supplementation. Data is mean ( $\pm$ SE) of three replicates and data followed by different letters denote significant difference at  $P \leq 0.05$ 

	Na		Ca	
	Leaf	Root	Leaf	Root
Control	3.866 $\pm$ 0.132c	4.493 $\pm$ 0.469c	5.908 $\pm$ 0.646b	3.420 $\pm$ 0.287b
NaCl	12.29 $\pm$ 1.119a	13.209 $\pm$ 0.339a	3.832 $\pm$ 0.139d	2.334 $\pm$ 0.141d
Ca	2.763 $\pm$ 195d	3.902 $\pm$ 0.105cd	7.813 $\pm$ 0.331a	4.990 $\pm$ 0.200a
NaCl + Ca	7.096 $\pm$ 0.193b	8.895 $\pm$ 0.573b	4.986 $\pm$ 0.126c	3.196 $\pm$ 0.176bc

under salinity conditions. Reduced photosynthetic efficiency due to salinity stress results from the increased chlorophyll degradation reflected in increased chlorophyllase activity and reduced synthesis of chlorophyll intermediates. Also, reduced photosynthetic efficiency due to salinity stress results from ROS induced photoinhibition primarily through damage to the structure of the pigment-protein complex (AHANGER et al., 2017). Optimal supplementation of Ca has adequately protected the pigment proteins and also have a visible impact on the allocation of key mineral ions like Mg and N for chlorophyll and Rubisco synthesis.

Further research is needed to unravel the actual mechanism.  $\text{Ca}^{2+}$  regulates photosynthetic electron transport and light-dependent metabolism through interplay with thylakoid acidification and redox homeostasis. Reduced NR activity due to salinity has been reported by others as well in crops like *Triticum aestivum* (AHANGER and AGARWAL, 2017) and *Vigna radiata* (SHAHI and SRIVASTAVA, 2018), however, Ca mediated regulation of NR activity under salinity has not been worked. Ca considerably ameliorated the salinity mediated decline in NR activity depicting its beneficial role in the protection of enzyme activity. Ca-mediated growth and photosynthetic improvement can be attributed to reduced Na uptake in them. Na is toxic to plants at higher concentrations and optimal Ca supplementation mediated decline in its accumulation directly shows its influence on the transport proteins controlling the uptake and the partitioning of Na within the cells mainly  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (DREVAL et al., 2005). Salinity affects the transport and homeostasis of Ca in plants by inhibiting  $\text{Ca}^{2+}$ -ATPase (GEISLER et al., 2000). Also, Ca availability may have changed the expression of other membrane proteins like tonoplast  $\text{H}^+$ -ATPase and  $\text{Na}^+/\text{H}^+$  antiport reducing the accumulation of excess levels of  $\text{Na}^+$ . Increased presence of Ca in cells is recognized by Ca sensors or  $\text{Ca}^{2+}$  binding proteins and leads to activation of protein kinases (TUTEJA and MAHAJAN, 2007).

Increased NaCl concentration in soil solution reduces the tissue growth by affecting the water content significantly and in confirmation to our results are the findings of (POLASH et al., 2018). Higher accumulation of sugars, Pro and GB in Ca supplemented seedlings resulted in enhanced RWC and also ameliorating the ill effects of salinity on cellular functioning. Calcium regulates the growth of plants by affecting mineral transport and accumulation of metabolites leading to the maintenance of water content (MOZAFARI, 2013). Ca-mediated enhancement in the accumulation of Pro, sugars, and GB resulted in higher RWC preventing the decline in photosynthesis and the hyper-osmotic effects of salinity on the protein structure (MUNNS and TESTER, 2008). BENHASSAINI et al. (2012) have also demonstrated salinity mediated enhancement in Pro in *Pistacia atlantica*. Pro and GB have a role in protecting the protein turnover machinery and improving the expression of stress proteins for greater tolerance. Greater accumulation of compatible osmolytes like Pro, GB, sugars improves plant performance under stressful conditions through increased RWC. Increased GB accumulation in Ca supplemented plants may have prevented salt mediated photo-inhibition by protecting the thylakoid membrane structure and the ATP synthesis leading to greater photosynthetic efficiency in them (WANG et al., 2018).

SIVAKUMAR et al (2000) has demonstrated increased carboxylase activity of Rubisco in plants accumulating higher contents of osmolytes. Pro and GB prevent the salinity mediated ROS initiated lipid peroxidation, deformation, and degradation of a nucleus, chromatin condensation and PCD. Ca supplementation enhanced the synthesis of Pro, sugars, and GB resulting in enhanced RWC, photosynthetic efficiency, and amelioration of oxidative damage. The osmolytes have been believed to strengthen the ROS scavenging systems and stress signaling mechanisms (AHANGER et al., 2018).

Salinity stress increased the generation of ROS like  $\text{H}_2\text{O}_2$  causing peroxidation of membranes, however, Ca supplementation reduced the generation of  $\text{H}_2\text{O}_2$  under normal conditions and also declined the NaCl induced ROS levels. Recently, AHANGER et al. (2018) have also demonstrated reduced membrane stability due to salinity mediated accumulation of  $\text{H}_2\text{O}_2$ . Stresses affect the polyunsaturated fatty acids significantly leading to greater membrane instability. The reduction in  $\text{H}_2\text{O}_2$  and mediated by Ca have been previously reported to impart a positive impact on the photosynthetic efficiency of plants (SAKHONWASEE and PHINGKASAN, 2017). Optimal supplementation of Ca may have reduced the activity of lipoygenase leading to prevention of any damage to membrane fatty acids besides its apparent effect on the efficiency of the antioxidant system. In *Vicia faba*, SIDDQUI et al. (2018) have also demonstrated up-regulation of the antioxidant system due to Ca supplementation under cadmium stress. In the present study, it was observed that Ca up-regulated antioxidant defense system by up-regulating SOD, APX and GR concomitant with an increase in the content of GSH, tocopherols, and AsA. Strengthening the antioxidant system prevents cellular oxidative damage by quick elimination of ROS. Ca-induced enhancement in an antioxidant system precludes any damage to chloroplast and mitochondrial electron transport chain by maintaining optimal  $\text{H}_2\text{O}_2$  levels so that their beneficial functions continue (MITTLER, 2017). Increased SOD activity in Ca supplemented seedlings prevent hydroxyl ( $\text{OH}^\cdot$ ) radical formation resulting in enhanced cellular functioning. Earlier AHMAD et al. (2015) have demonstrated up-regulation of the antioxidant system in mustard due to calcium supplementation. Increased APX and GR activity in Ca supplemented plants and maintenance of higher concentrations of non-enzymatic components lead to protection of cells from the oxidative burst through the support of cellular redox state. Antioxidants including low molecular weight antioxidants like AsA, GSH, and tocopherol are rich redox buffers, which interact with numerous cellular components besides other vital roles in defense and as enzyme cofactors influencing the growth and development of plants by modulating processes from mitosis and cell elongation to senescence and death (POTTERS et al., 2004). Ca supplementation strengthens the evidence that redox homeostasis involves precise ROS - antioxidant interaction as an interface for signals derived from the metabolism and the environment (FOYER and NOCTOR, 2005). The antioxidant system was further strengthened by the accumulation of phenols in Ca supplemented seedlings. Therefore, it is inferred from the present study that Ca availability potentially affects the growth and tolerance mechanisms in soybean and could be exploited for enhancing the yield productivity.

## Conclusion

It can be said that application of optimal Ca prevents the structural and functional integrity of soybean to counteract the salinity stress mediated growth restrictions. Optimal Ca availability benefited soybean under salinity stress by restricting the uptake of sodium and increasing the synthesis of photosynthetic pigments and photosynthetic functioning. Ca supplementation strengthened the indigenous tolerance mechanisms like the synthesis of compatible solutes and up-regulation of antioxidant system for preventing damage to cellular macromolecules. NaCl-mediated ROS-generated oxidative damage effects were averted by Ca availability by improving the redox components. By up-regulating the antioxidant system and the synthesis of osmolytes Ca availability can be exploited for improving the crop productivity. Further studies for unraveling the molecular mechanisms and the probable crosstalk of Ca with other key molecules in salinity stress mitigation can be handy.

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