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ORIGINAL PAPER

Calcium chloride and gibberellic acid protect linseed (*Linum usitatissimum* L.) from NaCl stress by inducing antioxidative defence system and osmoprotectant accumulation

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Abstract Salinity stress affects many metabolic facets of plants and induces anatomical and morphological changes resulting in reduced growth and productivity. To overcome the damaging effects of salinity, different strategies of the application of nutrients with plant hormones are being adopted. The present study was carried out with an aim to find out whether application of calcium chloride ($CaCl_2$) and gibberellic acid (GA_3) could alleviate the detrimental effects of salinity stress on plant metabolism. Fifteen days old plants were supplied with (1) 0 mM NaCl + 0 mg $CaCl_2$ kg^{-1} sand + 0 M GA_3 (control, T0); (2) 0 mM NaCl + 10 mg $CaCl_2$ kg^{-1} sand + 0 M GA_3 (T1); (3) 0 mM NaCl + 0 mg $CaCl_2$ kg^{-1} sand + 10^{-6} M GA_3 (T2); (4) 150 mM NaCl + 0 mg $CaCl_2$ kg^{-1} sand + 0 M GA_3 (T3); (5) 150 mM NaCl + 10 mg $CaCl_2$ kg^{-1} sand + 0 M GA_3 (T4); (6) 150 mM NaCl + 0 mg $CaCl_2$ kg^{-1} sand + 10^{-6} M GA_3 (T5); (7) 150 mM NaCl + 10 mg $CaCl_2$ kg^{-1} sand + 10^{-6} M GA_3 (T6). To assess the response of the crop to NaCl, $CaCl_2$ and GA_3, plants were uprooted randomly at 60 days after sowing. The presence of NaCl in the growth medium decreased all the growth and physio-biochemical parameters, except electrolyte leakage, proline (Pro) and glycine betaine (GB) content, thiobarbituric acid reactive substances (TBARS), H_2O_2 content, activities of superoxide dismutase (SOD) and catalase (CAT) and leaf Na content, which exhibited an increase of 37.6, 29.3, 366.9, 107.5, 59.1, 17.1, 28.4 and 255.2%, respectively, compared to the control plants. However, application of $CaCl_2$ in combination with GA_3 appears to confer greater osmoprotection by the additive role with NaCl in Pro and GB accumulation. Although the activities of antioxidant enzymes (SOD, CAT and POX) were increased by salt stress, the combined application of $CaCl_2$ and GA_3 to salt-stressed plants further enhanced the activities of these enzymes by 25.1, 6.7 and 47.8%, respectively, compared to plants grown with NaCl alone. The present study showed that application of $CaCl_2$ and GA_3 alone as well as in combination mitigated the adverse effect of salinity, but combined application of these treatments proved more effective in alleviating the adverse effects of NaCl stress.

Keywords Antioxidant · Calcium chloride · Gibberellic acid · Glycine betaine · Linseed · Proline

Abbreviations

CA	Carbonic anhydrase
CAT	Catalase
GB	Glycine betaine
g_s	Stomatal conductance
LRWC	Leaf relative water content
NR	Nitrate reductase
P_N	Net photosynthetic rate
POX	Peroxidase
Pro	Proline
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances

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LA	Leaf area per plant
SDW	Shoot dry weight per plant
RDW	Root dry weight per plant

Introduction

Soil salinity is one of the most serious environmental threats for plant survival and crop yield. It affects 19.5% of irrigated land and 2.1% dry land agriculture across the globe (FAO 2000). Salinity poses several undesirable effects on plants through hyperionic and hyperosmotic effects on several plant processes, leading to membrane disorganization, increase in reactive oxygen species (ROS) levels and metabolic toxicity (Hasegawa et al. 2000). High concentration of salts disturbs several biochemical processes and enzyme activities, particularly of CO_2 and nitrate assimilation. The enzyme carbonic anhydrase (CA) is found in abundance in the photosynthesizing tissues of both C_3 and C_4 plants and regulates the availability of CO_2 to ribulose bisphosphate carboxylase (rubisco) by catalyzing the reversible hydration of CO_2 (Badger and Price 1994). Whereas nitrate reductase (NR) is the enzyme that catalyses the first step of nitrate assimilation, which appears to be a rate-limiting process in the acquisition of nitrogen (Flores et al. 2002). Limited uptake of CO_2 results in decreased carbon reduction by Calvin cycle, which in turn leads to non-availability of oxidized $NADP^+$ for acceptance of electrons during photosynthesis, stimulating the formation of ROS such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (Peltzer et al. 2002). The toxic effects of O_2^- and H_2O_2 generate hydroxyl radicals and other destructive species such as lipid peroxides (Vaidyanathan et al. 2003).

Under stress, plants have evolved complex mechanisms to compete against these oxidative stresses by the synchronous action of various antioxidants. Of these, superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX) play significant roles in detoxifying ROS. SOD dismutates superoxide radicals to H_2O_2, whereas catalase and peroxidase are involved in converting H_2O_2 into water and oxygen. During salt stress, plants adapt to osmotic stress by accumulating some compatible solutes such as proline, GB, polyols and trehalose (Ghoulam et al. 2002; Sakamoto and Murata 2002). Proline plays a predominant role in protecting plants from osmotic stress. Thus, antioxidants and compatible solutes may provide a strategy to enhance salt tolerance in plants.

Linseed (*Linum usitatissimum* L.) is an important industrial crop for obtaining fibre (from stem) and oil (from seeds). But the susceptibility of the crop to salt stress is a major factor for reduced growth and productivity. The information is scanty on the tolerance of linseed plants to salinity. Therefore, enhancing salinity tolerance by some means would be an important strategy to improve the crop productivity.

To fulfil the demand of increasing population across the globe, it is highly desirable to alleviate the adverse effects of salt stress. Chemical treatments and agronomical crop management practices have been tried to alleviate the salinity stress with a little bit of success. A possible alternative is to induce the capability within plants to face successfully the detrimental situation by treatment with growth regulators such as gibberellins, which has been reported to mitigate the adverse effects of salinity by enhancing plant growth and yield. These also increase the N-use efficiency, NR and CA activities (Khan et al. 1996; Hayat et al. 2001), chlorophyll content and absorption of mineral nutrients (Afroz et al. 2005; Singh et al. 2005). One possible approach to reduce the effects of salinity on plant productivity is through the addition of mineral nutrients, of which calcium has a significant role in alleviating growth inhibition by salt in glycophytes (Yan et al. 1992). Calcium stabilizes cell membranes by connecting various proteins and lipids at membrane surfaces, influences the pH of cells and prevents solute leakage from cytoplasm (Hirschi 2004). Ca^{2+} sustains K^+transport and K^+/Na^+ selectivity in Na^+-challenged plants. The interaction of Na^+ and Ca^{2+} on plant growth is well established (Rengel 1992). However, meagre information is available on the efficacy of calcium and GA_3 in counteracting salinity stress in plants. Thus, we focused on $CaCl_2$ and GA_3 as possible inducers in the tolerance of linseed to salinity. The major objectives of our study were to determine the interactive efficiency of $CaCl_2$ and GA_3 treatments in restoring the metabolic alterations resulting from salt stress in linseed.

Materials and methods

Plant materials and growth conditions

A sand culture pot experiment was conducted under naturally illuminated environmental conditions. Healthy seeds of linseed (*L. usitatissimum* L.) were obtained from the Division of Oilseed Crops, CSA University of Agriculture and Technology, Kanpur, India. The earthen pots of 30-cm diameter, lined with polythene bag (to avoid contamination), were filled with 5 kg of sand. Before filling the pots, the sand was washed with 0.1 N HCl (to remove fungal contamination and nutrient cations), followed by tap and distilled water. The pots were arranged in a simple randomized design with four replicates. Prior to sowing, seeds

were surface sterilized with 1% sodium hypochlorite for 10 min, then vigorously rinsed with double distilled water (DDW) and sown in sand-filled pots supplied with Raukura's nutrient solution. After 2 weeks of sowing, thinning was done and four healthy plants of uniform size were maintained in each pot. Salinity treatment was given when the plants were at the stage of two to three true leaves. To avoid osmotic shock, NaCl concentration was increased gradually by 25 mM every 2 days until, from 0, a final concentration of 150 mM was reached. Calcium treatment was applied at the rate of 10 mg Ca kg^{-1} sand in 100 ml DDW. The source of calcium was calcium chloride ($CaCl_2$). A half dose of calcium (5 mg Ca kg^{-1} sand) was applied basally at the time of sowing and the remaining half dose was sprayed with GA_3 (10^{-6} M) after 2 weeks of NaCl treatment. By diluting with DDW, a 10^{-2} M stock solution of $GA_3$was prepared by dissolving GA_3 in ethyl alcohol. From this stock solution,10^{-6} M GA_3 was made using DDW. The plants supplied with DDW only were considered as control. To keep the sand moist, the experimental pots were irrigated daily with DDW (50–100 ml). The NaCl, $CaCl_2$ and GA_3 treatments were as follows:

0 mM NaCl + 0 mg $CaCl_2$ kg^{-1} sand + 0 M GA_3 T0 (control);
0 mM NaCl + 10 mg $CaCl_2$ kg^{-1} sand + 0 M GA_3 T1;
0 mM NaCl + 0 mg $CaCl_2$ kg^{-1} sand + 10^{-6} M GA_3 T2;
150 mM NaCl + 0 mg $CaCl_2$ kg^{-1} sand + 0 M GA_3 T3;
150 mM NaCl + 10 mg $CaCl_2$ kg^{-1} sand + 0 M GA_3 T4;
150 mM NaCl + 0 mg $CaCl_2$ kg^{-1} sand + 10^{-6} M GA_3 T5;
150 mM NaCl + 10 mg $CaCl_2$ kg^{-1} sand + 10^{-6} M GA_3 T6.

At every 2 days, 200 ml of Raukura's nutrient solution was added per pot. The salts used to make up the nutrient solution were as follows: macronutrient stock solution A (g l^{-1}) Mg $(NO_3)_2{\cdot}6H_2O$, 4.94; Ca $(NO_3)_2{\cdot}4H_2O$, 16.78; NH_4NO_3, 8.48; KNO_3, 2.28; and macronutrient stock solution B (g l^{-1}) KH_2PO_4, 2.67; K_2HPO_4, 1.64; K_2SO_4, 6.62; Na_2SO_4, 0.60; NaCl, 0.33. Micronutrient supplement (mg l^{-1}) H_3BO_3, 128.80; $CuCl_2{\cdot}2H_2O$, 4.84; $MnCl_2{\cdot}4H_2O$, 81.10; $(NH_4)_6$ $Mo_7O_{24}{\cdot}4H_2O$, 0.83; $ZnCl_2$, 23.45; ferric citrate pentahydrate, 809.84. The dilute solution applied to the plants was prepared by mixing 100 ml of each of the macronutrient stock solutions with 50 ml of the micronutrient supplement and diluting to 4.5 l using DDW. The pH was maintained at 6.0 by adding solution of H_2SO_4 or KOH.

The plants were sampled at 60 days after sowing. The performance of the crop was assessed in terms of plant height, leaf area per plant (LA), shoot dry weight per plant (SDW), root dry weight per plant (RDW), leaf relative water content (LRWC), activities of CA and NR, electrolyte leakage, net photosynthetic rate (P_N), stomatal conductance (g_s), leaf chlorophyll content, proline (Pro) content, glycine betaine (GB) content, thiobarbituric acid reactive substances (TBARS), hydrogen peroxide (H_2O_2) content, activities of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX) and leaf nitrogen (N), potassium (K), calcium (Ca) and sodium (Na) content, and K/Na and Ca/Na ratios.

Measurement of growth characteristics

The effect of salinity on growth parameters was studied in terms of plant height, LA, SDW and RDW.

Leaf area per plant was measured with the help of a sheet of graph paper. The area of three leaves (upper, middle and lower) of each plant from each treatment was determined using the graph paper. Dry weight of plants was recorded after drying the plants at 80°C for 24 h in a hot air oven.

Determination of physiological and biochemical parameters

Leaf relative water content was measured by adopting the method of Yamasaki and Dillenburg (1999). For each treatment, ten leaves were taken. To minimize the age effect, leaves were collected from the mid-section of the plant. To obtain their fresh mass (FM), the leaves were weighed just after removal from the stem. To determine turgid mass (TM), the leaves were kept in DDW inside a Petri dish covered with its lid for 4 h. Afterwards, the water was gently wiped out from the leaf surface with tissue paper and the leaves were weighed. To determine dry mass (DM), the leaf samples were dried at 80°C for 24 h. Values for FM, TM and DM were used to calculate LRWC using the equation below.

$$\mathrm{LRWC}(\%) = [(\mathrm{FM} - \mathrm{DM})/(\mathrm{TM} - \mathrm{DM})] \times 100$$

The activity of CA (E.C.4.2.1.1) enzyme was determined by the method of Dwivedi and Randhawa (1974). The leaf samples were cut into small pieces and suspended in cysteine hydrochloride solution. The samples were incubated at 4°C for 20 min. The pieces were blotted and transferred to the test tubes containing phosphate buffer (pH 6.8), followed by the addition of alkaline bicarbonate solution and bromothymol blue indicator. The test tubes were incubated at 5°C for 20 min. After addition of 0.2 ml of methyl red indicator, the reaction mixture was titrated against 0.05 N HCl. The results were expressed as μmol CO_2 kg^{-1}leaf fresh mass s^{-1}.

Nitrate reductase (E.C.1.6.6.1) activity was estimated by the intact tissue method of Jaworski (1971). Fresh leaf samples were weighed and transferred to plastic vials. To each vial, 2.5 ml phosphate buffer (pH 7.5), 0.2 M potassium nitrate and 5% isopropanol solutions were added. Each vial was incubated for 2 h in the dark at 30°C. To the incubated mixture, 1% sulphanilamide and 0.2% NED-HCl (*N*-1-naphthylethylene-diamine dihydrochloride) were added. The reaction mixture was kept for 20 min for maximum colour development. The absorbance was recorded at 540 nm using a spectrophotometer (SPEKOL-1500, Analytik Jena, Germany) and was compared with that of the calibration curve. The activity of NR was expressed as μmol NO_2 $h^{-1}g^{-1}$ of leaf fresh mass.

Electrolyte leakage was determined to assess membrane permeability as described by Lutts et al. (1995). The samples were washed three times with DDW to remove surface contamination. The leaf discs were prepared by cutting the young leaves using a punch plyer and were placed in a closed vial containing 10 ml of DDW and incubated on a rotatory shaker for 24 h and, subsequently, the electrical conductivity of the solution (EC_1) was determined. Samples were then autoclaved at 120°C for 20 min and the last electrical conductivity (EC_2) was noted after cooling the solution at room temperature. The electrolyte leakage was calculated as:

$$\text{Electrolyte leakage}(\%) = (EC_1/EC_2) \times 100$$

P_N and g_s were measured in cloudless clear days at light-saturating intensity between 11.00 and 12.00 hours on the uppermost, fully expanded leaves, using Infrared Gas Analyser (IRGA), LICOR-6200 portable photosynthesis system (LI-COR Lincoln, NE, USA).

Fresh leaf samples, taken from the youngest fully expanded leaves, were extracted with 80% acetone and the absorbance was recorded at 663 and 645 nm. The chlorophyll was determined by using the formula of Arnon (1949).

$$[(A_{645} \times 28.2) + (A_{663} \times 8.3)] \times [(v/1,000) \times W]$$

where W = weight (g) of the fresh leaves used for the extraction of pigment.

Pro content was determined spectrophotometrically adopting the ninhydrin method of Bates et al. (1973). As much as 300 mg of fresh leaf samples were homogenized in sulphosalicylic acid. To the extract, 2 ml each of acid ninhydrin and glacial acetic acid were added. The samples were heated to 100°C. The mixture was extracted with toluene and the free toluene was quantified spectrophotometrically at 528 nm using L-proline as a standard.

Glycine betaine content was estimated by the method of Grieve and Grattan (1983). Leaves were weighed and oven dried at 80°C and the dried leaves were finely ground with deionized water at 100°C for 60 min. GB concentration was determined at 365 nm, using aqueous extracts of dry-ground leaf material after reaction with KI_2–I_2.

Lipid peroxidation in leaves was determined by estimating TBARS according to the method of Heath and Packer (1968). The leaves were weighed and the homogenates containing 10% trichloroacetic acid and 0.65% thiobarbituric acid were heated at 95°C for 60 min, then cooled to room temperature and centrifuged at 10,000*g* for 10 min. The absorbance of the supernatant was read at 532 nm and corrected for non-specific turbidity by subtracting the absorbance of the same at 600 nm. The blank was 0.65% thiobarbituric acid in 10% trichloroacetic acid.

H_2O_2 content was determined according to Velikova et al. (2000). As much as 0.5 g of fresh leaf samples were homogenized with 5 ml of 0.1% (w/v) trichloroacetic acid. The homogenate was centrifuged at 12,000 rpm for 15 min and 0.5 ml of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M potassium iodide. The absorbance of supernatant was recorded at 390 nm. The content of H_2O_2 was calculated by comparison with a standard calibration curve, plotted by using different concentrations of H_2O_2.

Antioxidant enzyme assays

Leaf tissues were homogenized with three volumes (w/v) of an ice-cold extraction buffer [50 mM Tris–HCl, pH 7.8, 1 mM EDTA, 1 mM $MgCl_2$ and 1.5% (w/w) polyvinylpyrrolidone]. The homogenate was centrifuged at 15,000*g* at 4°C for 20 min. The supernatant was used as the crude extract for the assay of enzyme activities.

Superoxide dismutase (E.C.1.15.1.1) activity was determined according to Beauchamp and Fridovich (1971) by following the photoreduction of nitroblue tetrazolium (NBT). The reaction mixture contained 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 75 μM NBT, 2 μM riboflavin and 100 μl of the supernatant. Riboflavin was added as the last component and the reaction was initiated by placing the tubes under fluorescent lamps. The reaction was terminated after 10 min by removing the reaction tubes from the light source. Non-illuminated and illuminated reaction without supernatant served as calibration standards. The absorbance of the solution was measured at 560 nm.

The activity of CAT (E.C.1.11.1.6) was measured according to the method of Beers and Sizer (1952). The reaction mixture in a total volume of 3 ml contained 1 ml of 100 mM phosphate buffer (pH 7.0), 0.4 ml of 200 mM H_2O_2 and 0.1–0.2 ml of enzyme extract. The decrease in H_2O_2 was monitored at 240 nm.

POD (E.C.1.11.1.7) was assayed by the method of Upadhyaya et al. (1985). The reaction mixture contained

2.5 ml of 50 mM potassium phosphate buffer (pH 6.1), 1 ml of 1% hydrogen peroxide, 1 ml of 1% guaiacol and 10–20 µl of enzyme extract. The increase in absorbance at 420 nm was read.

Determination of leaf nutrient contents

Dried plant material was milled to pass through a 0.42 mm sieve, and 100 mg samples were analysed by a Kjeldahl method, which included 0.5% selenium as a catalyst and salicylic acid to reduce nitrate (Eastin 1978). The total N concentration was determined according to the method of Lindner (1944). Concentration of leaf K, Ca and Na was estimated using flame photometer (C150, AIMIL, India).

Statistical analysis

Each pot was treated as one replicate and all the treatments were repeated four times. The data were analysed statistically with SPSS-11 statistical software (SPSS Inc., Chicago, IL, USA). Mean was statistically compared by Duncan's multiple range test (DMRT) at $P < 0.05$% level.

Results

Plant height, LA, SDW and RDW were used to assess the adverse effects of salinity and supplementary $CaCl_2$ and /or GA_3 on plant growth.

Table 1 showed that the presence of NaCl in growth medium decreased all the growth attributes. Under saline conditions, the above growth parameters decreased by 12.4, 22.7, 16.9 and 29.2%, respectively, as compared to the control. Treatment with $CaCl_2$ or GA_3 overcame the adverse effects of salinity, but their combined application was more effective compared to their individual application. The values recorded from salt-stressed plants supplied with $CaCl_2$ and GA_3 together were at par with that of control plants for plant height, SDW and RDW (Table 1).

Presence of NaCl in the growth medium induced significant decrease in LRWC (Fig. 1a), whereas maximum LRWC was recorded in unstressed plants treated with $CaCl_2$ or GA_3. Although application of $CaCl_2$ or GA_3 alone did not show a significant change in LRWC of NaCl-stressed plants, the combined application exhibited a significant increase of 22.5% in LRWC when compared with the plants exposed to NaCl only (Fig. 1a).

The NaCl-generated stress suppressed the activities of CA and NR (Fig. 1b, c). But a different pattern of crop response was observed when these two parameters were studied in $CaCl_2$ and GA_3-treated plants in the presence or absence of NaCl. Under non-saline and saline conditions, $CaCl_2$ or GA_3 increased CA and NR activities. However, the subsequent treatment of the stressed plants with the combination of $CaCl_2$ and GA_3 neutralized the effect of NaCl and caused a considerable improvement in the activities of these two enzymes compared to their individual application (Fig. 1b, c).

The plants supplemented with NaCl exhibited a significant increase in electrolyte leakage compared to the plants grown under non-saline medium. $CaCl_2$ and GA_3 decreased electrolyte leakage in the plants grown with or without NaCl. However, combined application of $CaCl_2$ and GA_3 to the NaCl-exposed plants was found to be more effective in the reversal of the adverse effect of NaCl stress and caused a significant decrease in electrolyte leakage (Fig. 1d).

The plants exposed to NaCl stress exhibited reduction in P_N and g_s when compared with control (Fig. 2a, b). On the other hand, an increase in these parameters was recorded in the plants supplied with $CaCl_2$ and GA_3 alone. Similar inhibitory action on the effect of salt stress was recorded when $CaCl_2$ was applied in association with GA_3 and this combination proved more effective for the reversal of

Table 1 Effect of calcium chloride and gibberellic acid on growth parameters of linseed grown under NaCl stress

Treatments	Plant height (cm)	Leaf area per plant (cm^2)	Shoot dry weight (g)	Root dry weight (g)
T0 (control)	54.1 ± 1.27^{c}	142.78 ± 1.69^{b}	3.79 ± 0.05^{b}	0.48 ± 0.01^{b}
T1	58.7 ± 1.36^{b}	160.41 ± 2.72^{a}	4.06 ± 0.07^{a}	0.56 ± 0.02^{a}
T2	62.7 ± 0.43^{a}	155.65 ± 1.52^{a}	4.17 ± 0.06^{a}	0.58 ± 0.01^{a}
T3	47.4 ± 0.78^{e}	110.35 ± 1.16^{e}	3.15 ± 0.03^{d}	0.34 ± 0.02^{d}
T4	49.6 ± 0.42^{de}	119.33 ± 2.02^{d}	3.29 ± 0.05^{cd}	0.40 ± 0.01^{c}
T5	52.9 ± 1.36^{cd}	121.97 ± 1.94^{d}	3.42 ± 0.05^{c}	0.42 ± 0.03^{c}
T6	55.4 ± 0.98^{bc}	135.44 ± 1.93^{c}	3.67 ± 0.06^{b}	0.48 ± 0.03^{b}

Each value is mean of four replicates ($n = 4$) ± SE. Data with the same letter within a column are not significantly different at $P < 0.05$ level as determined by Duncan's multiple range test [0 mM NaCl + 0 mg $CaCl_2$ + 0 M GA_3 (control, T_0), 0 mM NaCl + 10 mg $CaCl_2$ + 0 M GA_3 (T1), 0 mM NaCl + 0 mg $CaCl_2$ + 10^{-6} M GA_3 (T2), 150 mM NaCl + 0 mg $CaCl_2$ + 0 M GA_3 (T_3), 150 mM NaCl + 10 mg $CaCl_2$ + 0 M GA_3 (T_4), 150 mM NaCl + 0 mg $CaCl_2$ + 10^{-6} M GA_3 (T5), 150 mM NaCl + 10 mg $CaCl_2$ + 10^{-6} M GA_3 (T6)]

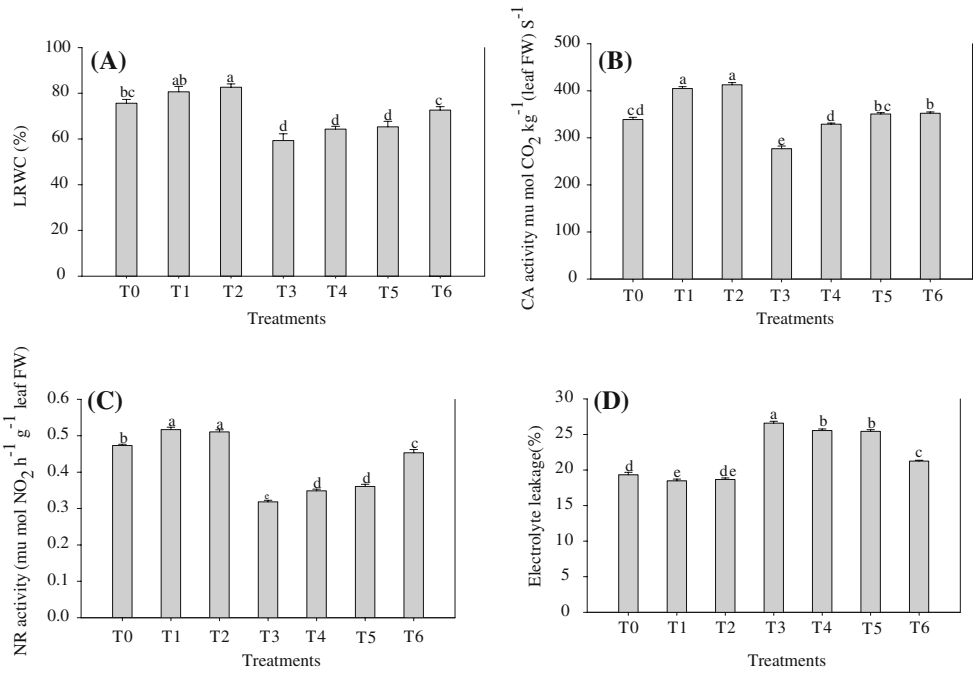

Fig. 1 Effect of calcium chloride and gibberellic acid on LRWC, activities of CA and NR and electrolyte leakage of linseed grown under NaCl stress. Each value represents the mean of four replicates with S.E. determined. The *different and same letters* mean significant ($P < 0.05$). [0 mM NaCl + 0 mg $CaCl_2$ + 0 M GA_3 (control, T_0), 0 mM NaCl + 10 mg $CaCl_2$ + 0 M GA_3 (T1), 0 mM NaCl + 0 mg $CaCl_2$ + 10^{-6} M GA_3 (T2), 150 mM NaCl + 0 mg $CaCl_2$ + 0 M GA_3 (T_3), 150 mM NaCl + 10 mg $CaCl_2$ + 0 M GA_3 (T_4), 150 mM NaCl + 0 mg $CaCl_2$ + 10^{-6} M GA_3 (T5), 150 mM NaCl + 10 mg $CaCl_2$ + 10^{-6} M GA_3 (T6)]

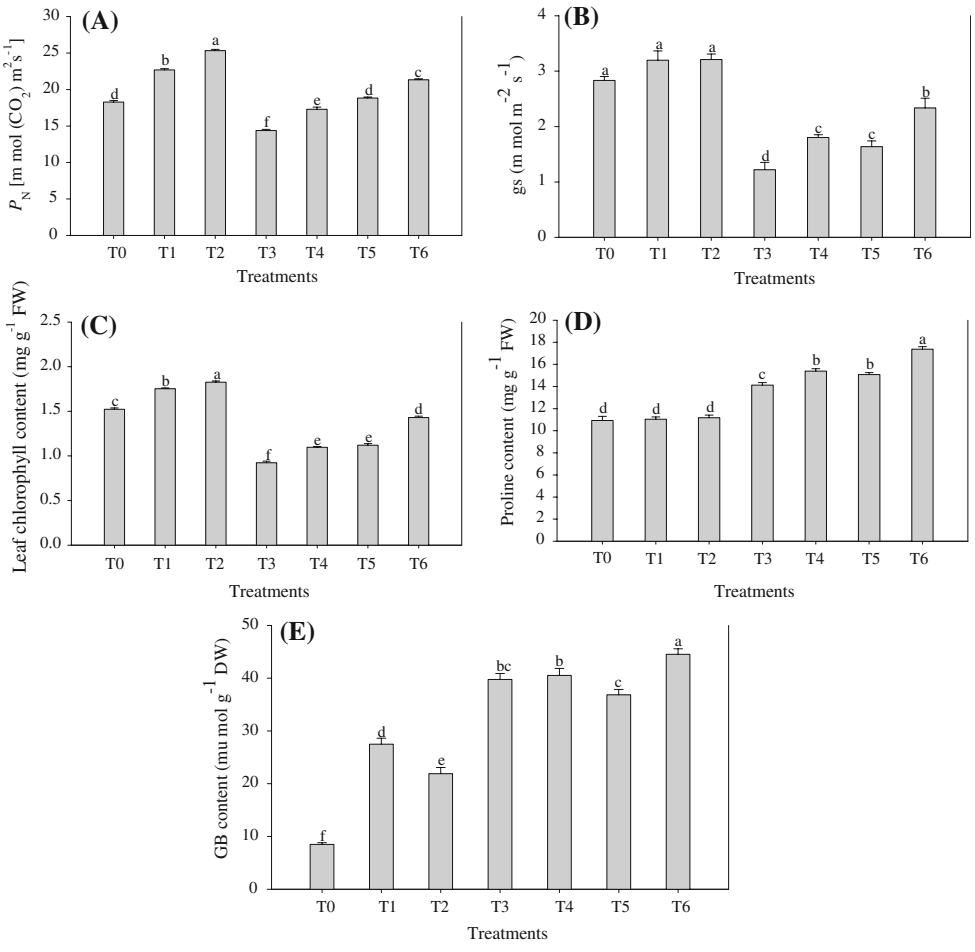

Fig. 2 Effect of calcium chloride and gibberellic acid on P_N, g_s, leaf chlorophyll content and proline and GB content of linseed grown under NaCl stress. Each value represents the mean of four replicates with S.E. determined. The *different and same letters* mean significant ($P < 0.05$). [0 mM NaCl + 0 mg $CaCl_2$ + 0 M GA_3 (control, T_0), 0 mM NaCl + 10 mg $CaCl_2$ + 0 M GA_3 (T1), 0 mM NaCl + 0 mg $CaCl_2$ + 10^{-6} M GA_3 (T2), 150 mM NaCl + 0 mg $CaCl_2$ + 0 M GA_3 (T_3), 150 mM NaCl + 10 mg $CaCl_2$ + 0 M GA_3 (T_4), 150 mM NaCl + 0 mg $CaCl_2$ + 10^{-6} M GA_3 (T5), 150 mM NaCl + 10 mg $CaCl_2$ + 10^{-6} M GA_3 (T6)]

altered P_N and g_s than their individual application to NaCl-suffered plants (Fig. 2a, b). The presence of NaCl caused a significant decrease in chlorophyll content compared to the control. Under stress-free medium, maximum content was found in the plants which were subjected to GA_3 alone. However, under salt stress maximum values for chlorophyll content were recorded in the plants, supplied with the combination of $CaCl_2$ and GA_3 treatments (Fig. 2c).

It is evident from Fig. 2d that Pro was higher in NaCl-treated plants compared to the control. Application of $CaCl_2$ and GA_3 individually on unstressed plants could not bring about a significant change from control in the level of Pro. However, in association with NaCl, they alleviated the quantity of Pro. The highest level of Pro was found in salt-stressed plants, which were subjected to both $CaCl_2$ and GA_3. GB content showed a significant increase in NaCl-suffered plants, compared to the control (Fig. 2e). The level of GB content was lower in unstressed plants supplied with $CaCl_2$ or GA_3 alone when compared with salt-stressed plants. On the contrary, the level was higher when it was compared with the control plants, which gave the lowest value for GB content. Application of $CaCl_2$ and GA_3 together to the saline growth medium triggered the plants to accumulate more GB content than $CaCl_2$ and GA_3 individually (Fig. 2e).

Oxidative damage to tissue lipid was assessed by the content of total TBARS. The NaCl-generated stress has a promotive effect on TBARS (Fig. 3a). Application of $CaCl_2$ to NaCl-suffered plants decreased TBARS content than the plants grown with NaCl alone, whereas, GA_3 treatment had no significant effect on this parameter in NaCl-treated plants. The maximum alleviating effect on the salt stress was recorded in the plants, which received $CaCl_2$ and GA_3 both, as reflected by 18.7% decrease in TBARS compared with salt-stressed plants (Fig. 3a).

Figure 3b exhibits that plants grown with NaCl accumulated maximum H_2O_2 content. In contrast, lowest values for H_2O_2 content were recorded in the non-stressed plants supplied with $CaCl_2$ or GA_3 only. Application of $CaCl_2$ alone to salt-stressed plants reduced H_2O_2 content, but GA_3 could not alleviate the adverse effect of NaCl in this parameter. Whereas, combination of $CaCl_2$ and GA_3 arrested the effect of salt stress up to a considerable limit and exhibited 19.0% decrease in H_2O_2 content compared with the plants grown with NaCl only (Fig. 3b).

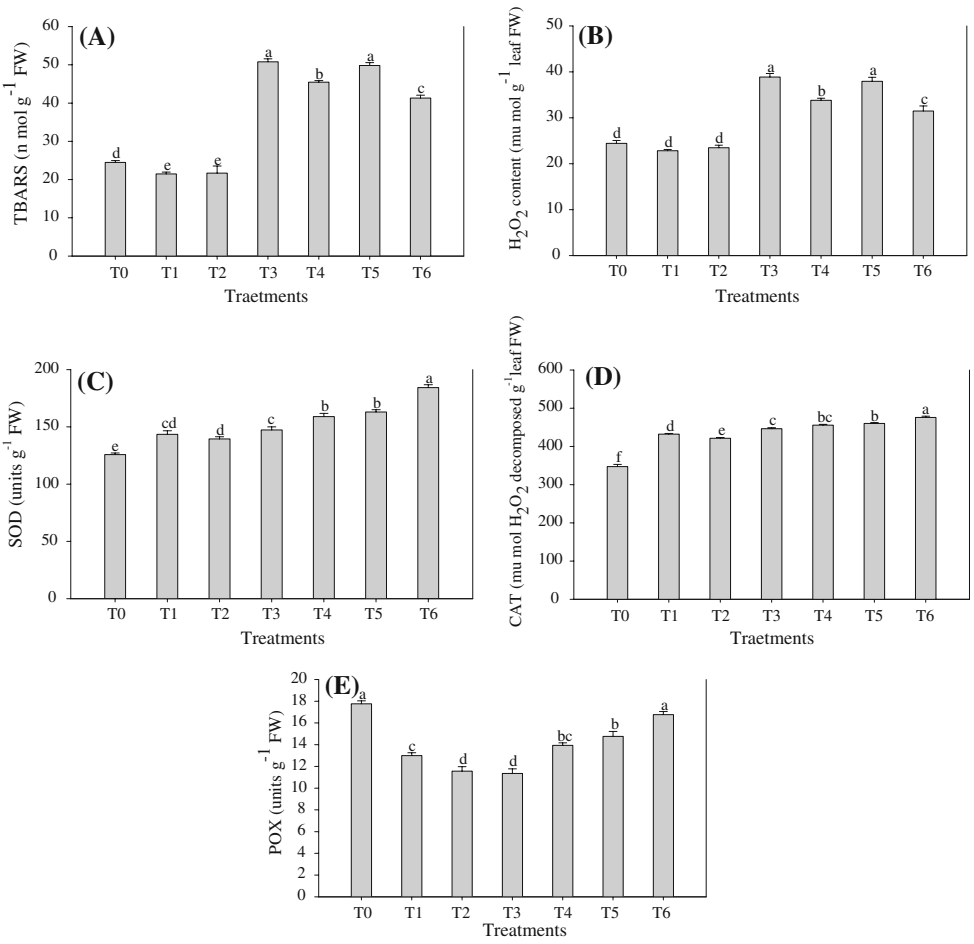

Fig. 3 Effect of calcium chloride and gibberellic acid on TBARS, H_2O_2 content and activities of SOD, CAT and POX of linseed grown under NaCl stress. Each value represents the mean of four replicates with S.E. determined. The *different and same letters* mean significant ($P < 0.05$). [0 mM NaCl + 0 mg $CaCl_2$ + 0 M GA_3 (control, T_0), 0 mM NaCl + 10 mg $CaCl_2$ + 0 M GA_3 (T1), 0 mM NaCl + 0 mg $CaCl_2$ + 10^{-6} M GA_3 (T2), 150 mM NaCl + 0 mg $CaCl_2$ + 0 M GA_3 (T_3), 150 mM NaCl + 10 mg $CaCl_2$ + 0 M GA_3 (T_4), 150 mM NaCl + 0 mg $CaCl_2$ + 10^{-6} M GA_3 (T5), 150 mM NaCl + 10 mg $CaCl_2$ + 10^{-6} M GA_3 (T6)]

The SOD activity was increased by NaCl, $CaCl_2$ and GA_3 treatments, when compared to the control plants. Being equally effective, $CaCl_2$ or GA_3 separately enhanced SOD activity compared to the plants grown with NaCl only (Fig. 3c). The NaCl-suffered plants supplemented with both $CaCl_2$ and GA_3 showed further increase in the enzyme activity and the value recorded from this treatment was 25.1% higher than the plants grown with NaCl only (Fig. 3c).

Activity of CAT was increased by NaCl, $CaCl_2$ and GA_3 compared with the control. A significant increase in CAT activity was recorded with GA_3 which showed parity with $CaCl_2$ under saline medium. As in the case of SOD, CAT also came out with enhanced activity in the salt-stressed plants, which were supplemented with the combination of $CaCl_2$ and GA_3 when compared with the plants subjected to NaCl only (Fig. 3d).

Figure 3e shows decreased values for POX activity in $CaCl_2$, GA_3 and NaCl-treated plants when compared with the control, whereas the reverse was true when $CaCl_2$-treated plants were compared with salt-stressed plants. $CaCl_2$ and GA_3 being equally effective enhanced POX activity in NaCl-stressed plants than the plants grown with NaCl only. Similar to SOD and CAT, POX activity was also accelerated and the adverse effect of salinity was effectively inhibited by the combined application of $CaCl_2$ and GA_3 to NaCl-stressed plants. The value recorded from this treatment was statistically at par with that of control (Fig. 3e).

The results exhibited that NaCl-fed plants gave the lowest nutrient (leaf N, K and Ca) concentration, but maximum Na concentration (Fig. 4a–d). Application of $CaCl_2$ and GA_3 individually was found to be effective in improving leaf N, K and Ca concentration and decreasing leaf Na concentration, but efficiency of the combined $CaCl_2$ and GA_3-treated plants was better in alleviating the adverse effect of salinity stress on these nutrients. The K/Na and Ca/Na ratios were found to be markedly low in NaCl-fed plants. However, maximum ratio for these two parameters was found in $CaCl_2$-treated plants grown under stress-free medium. Application of $CaCl_2$ in association with GA_3 mitigated the adverse effect of salinity and

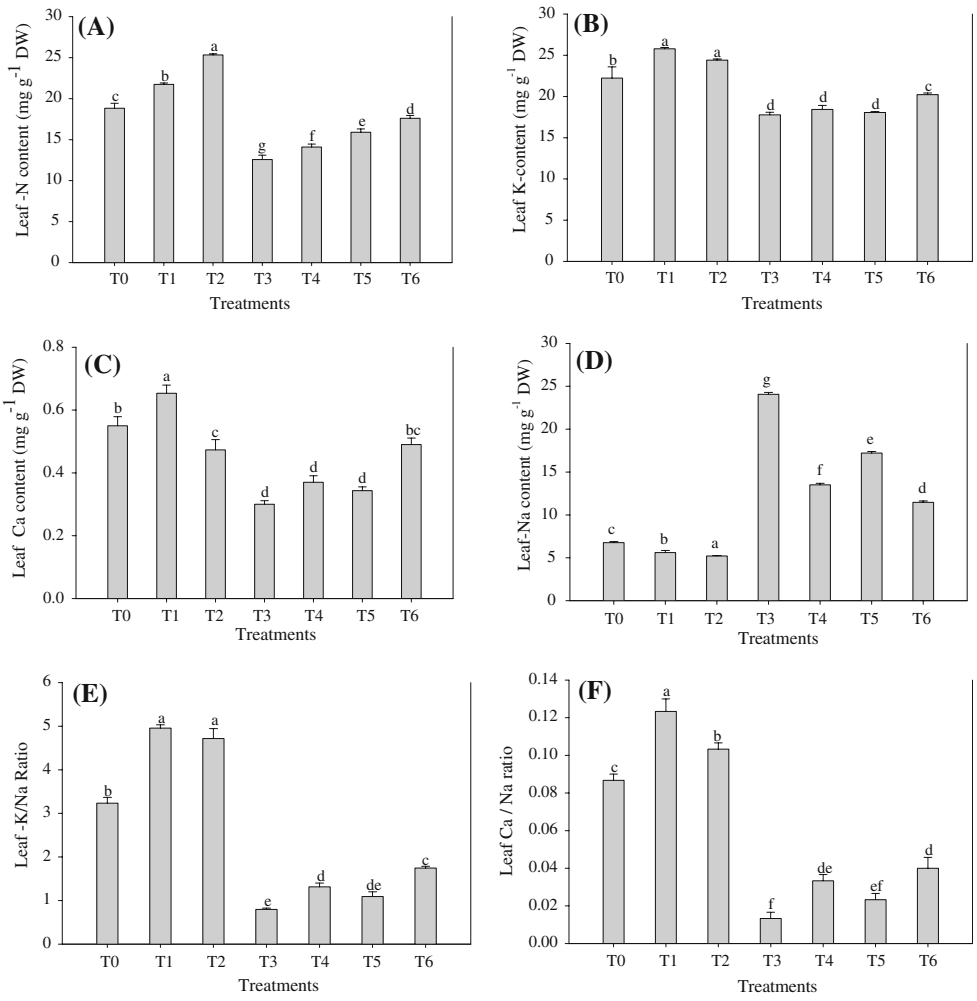

Fig. 4 Effect of calcium chloride and gibberellic acid on Leaf N, K, Ca and Na content and leaf K/Na ratio and leaf Ca/Na ratio of linseed grown under NaCl stress. Each value represents the mean of four replicates with S.E. determined. The *different and same letters* mean significant ($P < 0.05$). [0 mM NaCl + 0 mg $CaCl_2$ + 0 M GA_3 (control, T_0), 0 mM NaCl + 10 mg $CaCl_2$ + 0 M GA_3 (T1), 0 mM NaCl + 0 mg $CaCl_2$ + 10^{-6} M GA_3 (T2), 150 mM NaCl + 0 mg $CaCl_2$ + 0 M GA_3 (T_3), 150 mM NaCl + 10 mg $CaCl_2$ + 0 M GA_3 (T_4), 150 mM NaCl + 0 mg $CaCl_2$ + 10^{-6} M GA_3 (T5), 150 mM NaCl + 10 mg $CaCl_2$ + 10^{-6} M GA_3 (T6)]

improved K/Na and Ca/Na ratios significantly than their indivdual application (Fig. 4e, f). However, maximum ratio for these two parameters was recorded in $CaCl_2$-treated plants grown under stress-free medium. Addition of $CaCl_2$ or GA_3 to NaCl-suffered plants equally improved K/Na and Ca/Na ratio compared to the plants grown with NaCl only. Application of $CaCl_2$ in association with GA_3 mitigated the adverse effect of salinity and further improved K/Na and Ca/Na ratios significantly than their individual application (Fig. 4e, f).

Discussion

The results reported in the present study exhibited that $CaCl_2$ and GA_3 applied individually as well in combination helped the salinity-challenged plants to a different degree in the reversal of altered growth and physio-biochemical processes in linseed.

Addition of NaCl to the growth medium resulted in general reduction in the growth of linseed plants in terms of plant height, LA, SDW and RDW (Table 1). The inhibition in growth parameters by salt stress was also reported by Meloni et al. (2004) in *Prosopis alba*, Singh et al. (2007) in rice and Siddiqui et al. (2009) in *Brassica juncea*. The foliar application of GA_3 with or without $CaCl_2$ was found to be effective in alleviating the adverse effect of salt stress and improved plant height. The increase in plant growth as a response to $CaCl_2$ and GA_3 occurs as a consequence of cell elongation and cell division (Tanimoto 1990; Hirschi 2004). The combined effect of $CaCl_2$ and GA_3 on the growth characteristics was found to be more effective and, consequently, roots grown as well, caused in turn an increase in total dry matter production (Table 1).

The present work reveals that NaCl stress caused a significant reduction in LRWC and enzyme activities of CA and NR and significant increase in electrolyte leakage. The decreased value for LRWC may originate from salinity-induced constraints on water availability and water uptake, thus resulting in the reduction of root pressure-driven xylem transport rates of water and mineral nutrients to the shoot. Consequently, less water is available for normal growth and development as shown by low values of LRWC (Fig. 1a). Decreased value of LRWC under salt stress was also reported by Khan et al. (2007) and Cabañero et al. (2004). However, NaCl-stressed plants in association with $CaCl_2$ and GA_3 significantly enhanced LRWC. These treatments reduced membrane injury by dehydration and improved the water status of plants. This may be the reason for improved growth of seedlings under $CaCl_2$ and GA_3 treatments. In fact, calcium in growth medium competes with Na^+ ions for membrane binding sites and is essential for root elongation (Sumner 1993; Kinraide 1998), resulting in more efficient water and nutrient uptake under saline conditions, as exhibited by enhanced level of LRWC and leaf nutrient contents (Fig. 1a, 4a–f).

Carbonic anhydrase is the enzyme that plays many diverse roles in physiological processes such as ion exchange, acid–base balance, carboxylation/decarboxylation reactions and inorganic carbon diffusion between the cell and its environment as well as within the cell (Badger and Price 1994; Georgios et al. 2004). NaCl-fed plants showed the lowest CA activity (Fig. 1b), which may be due to inactivation of rubisco (Soussi et al. 1998), which sequentially reduces the net photosynthetic rate, carbon metabolism, leaf chlorophyll content and photosynthetic efficiency (Seeman and Critchley 1985). In contrast, combined application of $CaCl_2$ and GA_3 improved CA activity in the plants grown with NaCl. The enhanced CA activity might have helped in the reversible hydration of CO_2 and maintained its constant supply to rubisco, which may be one of the reasons behind the improved P_N in $CaCl_2$ and GA_3-treated plants (Fig. 2a).

Nitrate reductase is the rate-limiting enzyme in nitrogen assimilation and a key point of metabolic regulation. It is noteworthy that NaCl-exposed plants showed significant decrease in NR activity (Fig. 1c). This result strengthens the findings of Garg et al. (1997), Gratten and Grieve (1994), Nathawat et al. (2005) and Siddiqui et al. (2009), who reported that salinity causes decrease in N uptake and in the activity of nitrogen assimilation enzymes. It was noted that combined application of $CaCl_2$ and GA_3 mitigated the adverse effect of salt stress and enhanced the NR activity. The activity of NR is highly variable and depends on the presence of hormones such as gibberellic acid and/or cytokinin (Roth-Bejerano and Lips 1970).

The plants treated with NaCl exhibited a significant increase in electrolyte leakage compared to the control plants. Similar results were reported by Lutts et al. (1996), Khan et al. (2007) and Siddiqui et al. (2008). Salt-stressed plants supplied with $CaCl_2$ and GA_3 alone or in combination, showed a significant decrease in electrolyte leakage compared to the plants grown with NaCl alone (Fig. 1d). A reduction in electrolyte leakage may be explained on the basis of the role of calcium in controlling membrane structure and function. Calcium, by binding to phospholipids stabilizes lipid bilayers and thus provides structural integrity to cellular membranes.

Salt-fed plants exhibited a significant reduction in P_N, g_s and chlorophyll content (Fig. 2a–c). The degree of reduction in P_N and g_s under salt stress might be due to the closure of stomata caused by excessive accumulation of Na^+ ion in the guard cells, which reduces the availability of internal CO_2. These results are in agreement with the findings of Thiel and Blatt (1991). Furthermore, decreased

chlorophyll content might be due to instability of protein complexes and destruction of chlorophyll by increased activity of chlorophyll-degrading enzyme chlorophyllase under stress condition (Reddy and Vora 1986). Moreover, NaCl also inhibits the activities of key enzymes of photosynthesis, namely rubisco and PEP carboxylase (Soussi et al. 1998), and consequently, reduction in stomatal conductance and CA activity. Interestingly, it is cleared from our findings that application of $CaCl_2$ and GA_3 alone as well as in combination helped to a different degree the reversal of altered P_N, g_s and chlorophyll concentration induced by salt stress (Fig. 2a–c). The increase in P_N and g_s by $CaCl_2$ and GA_3 may be explained on the basis of the role of combination of treatment in the accumulation of organic solutes, i.e. Pro and GB, increase in leaf nutrient concentration, LRWC, activities of CA, NR, SOD, CAT and POX. The cumulative response of these parameters might have provided an environment to the plants to perform photosynthesis normally, which is further confirmed by enhanced dry matter production (Table 1).

Osmotic adjustment is the main part of the physiological machinery by which plants respond to salt stress. Accumulation of organic solutes (e.g. Pro and GB) in the cytosol and other organelles help in the osmotic adjustment of plants. In response to salt stress, a large group of plants accumulated higher content of organic solutes in their tissues. Among the organic solutes, Pro and GB content exhibited increased accumulation in plants grown with NaCl alone, when compared with the control (Fig. 2d, e). GB stabilizes the quaternary structures of complex proteins, such as in PS-II (Saneoka et al. 1995). Application of $CaCl_2$ and GA_3 alone or in combination increased Pro and GB content significantly (Fig. 2d, e). The enhanced level of Pro under salt stress might have been caused by the induction or activation of Pro biosynthesis or decrease in oxidation of Pro to glutamate or decrease in its utilization in protein synthesis or enhancement in protein turnover. Thus, Pro may be the major source of energy and nitrogen during immediate post-stress metabolism and accumulated Pro apparently supplies energy for growth and survival, thereby inducing salinity tolerance. Higher accumulation of GB in $CaCl_2$ and GA_3-treated plants counteracted the ill effect of salinity and might have helped in the osmotic adjustment of plants. Thus, application of $CaCl_2$ in association with GA_3 enhanced GB content and improved the plant's capacity of tolerance to salt stress, which was witnessed by increase in P_N, g_s and activities of CA and NR.

Under salt stress, plants are overloaded with ROS, which inhibit several plant processes and cause damage to the plants in different ways. ROS generate hydroxyl radicals and other destructive species such as lipid peroxides (Vaidyanathan et al. 2003), which causes destruction of cell membrane as reflected by increased values for TBARS under salt stress (Fig. 3a). Thus, to maintain metabolic functions under stress, the scavenging of ROS is required. ROS scavenging depends on detoxification mechanism provided by antioxidant enzymes (SOD, CAT, POX). Under salt stress, plants not only exhibited increased level of TBARS, but also H_2O_2 content and activities of antioxidative enzymes (SOD, CAT and POX), compared to their respective controls (Fig. 3a–e). Tuna et al. (2008) also reported a similar effect of gibberellic acid on the levels of antioxidants. However, when NaCl-fed plants were supplied with $CaCl_2$ and/or GA_3, decreased TBARS and H_2O_2 concentration were observed (Fig. 3a, b). On the other hand, combined application of $CaCl_2$ and GA_3 to salt-stressed plants improved the activities of the above-mentioned antioxidative enzymes. GA_3 is known to enhance the uptake of Ca^{2+} into the cytoplasm (Moll and Jones 1981). The accumulated Ca^{2+} might have been responsible for decreased TBARS and H_2O_2 content, as the unique importance of Ca^{2+} for stabilization of membranes is well known (Marschner 2002; Hirschi 2004). Increased value for membrane permeability was recorded with NaCl application, and this increase was decreased by added calcium (Kaya et al. 2002).

A reduction in leaf nutrient contents (N, K and Ca) and a significant increase in leaf Na content were recorded in the plants grown with NaCl alone (Fig. 4a–d). Similar results were reported by Siddiqui et al. (2008, 2009). The important control mechanism under salt stress is the higher K/Na and Ca/Na selectivity in plants. It has been suggested that this is an important selection criterion for salt tolerance (Ashraf 2002; Wenxue et al. 2003). In this study, K/Na and Ca/Na ratios were the most sensitive parameters to salinity and a similar trend was observed with respect to N, K and Ca concentration in the leaves. Under salt stress, high concentration of N, K, Ca and K/Na and Ca/Na ratio was recorded when plants were provided combined $CaCl_2$ and GA_3 treatment, as compared to their individual application. The enhanced accumulation of N, K and Ca by the application of $CaCl_2$ and GA_3 individually as well as in combination have been major factors for increasing dry matter production and plant height, because they are components of many metabolically active compounds and play an important role in physiological and biological functions (Marschner 2002). Siddiqui et al. (2008) also observed an increase in nutrient concentration of salt-stressed plants treated with GA_3. Moreover, calcium is known to play many important roles in the physiology of crop plants. It influences availability and uptake of nutrients and also enhances nitrogen use efficiency (Easterwood 2002). It appears that increased leaf K concentration enhanced photosynthetic efficiency possibly by increasing the number of chloroplast per cell, number of cells per leaf and

consequently increased leaf area. Increased K/Na and Ca/Na ratio due to application of $CaCl_2$ to salinity-affected plants may be explained on the basis of the property of Ca to replace Na, as a result of mutual competition between the two ions for a transport site on a carrier protein (Epstein 1962; Cramer 1992), and a decrease in the magnitude of the pH gradient across the plasma membrane resulting in net reduced influx of Na and inhibition of membrane-associated carrier protein thereby maintaining its integrity (Colmer et al. 1994). The present work indicates that salt tolerance is not only associated with increase in the concentration of N, K and Ca, but also with high ratio of K/Na and Ca/Na by the application of $CaCl_2$ and GA_3 individually as well as in combination.

Conclusion

The assessment of the results allows us to conclude that application of $CaCl_2$ and GA_3 increased P_N, g_s and chlorophyll content with a parallel increase in enzyme activities and nutrient accumulation that may be responsible for enhanced dry matter production in salt-stressed plants. However, combined application of $CaCl_2$ and GA_3 reversed the inhibitory effect of salt stress and adjusted the plants to perform normally by reducing the membrane damage and lipid peroxidation, which is brought about by the induction of antioxidative enzymes and osmoprotectants accumulation.

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