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Exogenous calcium alters pigment composition, γ-glutamyl kinase and proline oxidase activities in salt-stressed *Withania somnifera*

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Abstract

In the present study effect of sodium chloride and calcium chloride on the proline metabolism of *Withania somnifera* plants was studied. The plants were treated with 100 mM NaCl, 5 mM $CaCl_2$, 100 mM NaCl with 5 mM $CaCl_2$ solutions. Ground water was used for irrigation to control plants. Plants were harvested randomly on 30 and 50 days after sowing (DAS). NaCl and $CaCl_2$ stressed plants showed decreased chlorophyll 'a', 'b', total chlorophyll and carotenoid content and also proline oxidase activity and increased γ-glutamyl kinase activity when compared to control. The NaCl with $CaCl_2$ treated plants increased chlorophyll 'a', 'b', total chlorophyll content and proline oxidase activity and decreased the γ-glutamyl kinase activity in all parts of *Withania somnifera* when compared to NaCl treated plants.

Keywords: calcium chloride; pigments; proline metabolizing enzymes; sodium chloride; *Withania somnifera.*

Abbreviations: DAS_days after sowing; FYM_farmyard manure; GAS_glutamic γ-semialdehyde; P5C_pyroline-5-carboxylate; PMS_phenazine methosulphate; DCPIP_2,6-dichlorophenol indophenol; NaCl_sodium chloride; SD_standard deviation; $CaCl_2$_calcium chloride; OD_optical density.

Introduction

Soil salinity is an important determinant of plant growth and survival and exposure to salt leads to increased maintenance and costs for plant cells (Jaleel et al., 2007a). Abiotic stress environment can induce a wide number of responses in plants ranging from readjustments of transport and metabolic processes leading to growth inhibition (Jaleel et al., 2007b, 2008a,b). Soil salinity inhibits plant growth and agricultural productivity. Excessive sodium (Na^+) inhibits growth of many salt sensitive plants and glycophytes, which includes most crop plants. A variety of mechanisms contribute to salt tolerance (Jaleel et al., 2007c).

The enzymes γ-glutamyl kinase and γ-glutamyl phosphate reductase are regarded as an enzyme complex called pyroline-5-carboxylate (P5C) synthetase because the resulting product, glutamic γ-semialdehyde (GAS) is non-enzymatically converted to P5C. The regulation of proline synthesis is probably controlled by the activity of P5C synthase (Boggess et al., 1976; Jaleel et al., 2007d). Proline oxidase converts proline to glutamate. Thus this

enzyme also influences the level of proline. Proline accumulation in salt stressed cells may also occur by decreased oxidation to glutamic acid (Jaleel et al., 2007e). Such reduction in proline oxidase activity and simultaneous increase in proline level also occurred following low temperature and drought (Jaleel et al., 2007f). Supplementing the medium with Ca^{2+} alleviates growth inhibition by salt of glycophytic plants (Jaleel et al., 2007g). Ca^{2+} sustains K^+ transport and K^+/Na^+ selectivity in Na^+ challenged plants (Jaleel et al., 2008b).

Withania somnifera Dunal, known as ashwagandha, has been an important herb in the Ayurvedic and indigenous medical systems for centuries in India (Jaleel et al., 2008c). In view of its varied therapeutic potential, it is the subject of considerable modern scientific attention (Jaleel et al., 2008d). A perusal of the literature showed that there is a lack of information on the responses of this plant to combined effects of salinity from NaCl and $CaCl_2$. The present investigation was therefore undertaken to study the interactive effects of NaCl and $CaCl_2$ on the photosynthetic pigments and proline metabolising enzymes like γ-glutamyl kinase and proline oxidase in *Withania somnifera*.

Materials and methods

Plant materials and NaCl treatments

Seeds of *Withania somnifera* were surface sterilized with 0.2 per cent $HgCl_2$ solution for 5 minutes with frequent shaking and then thoroughly washed with deionised water. The seeds were sown in plastic pots (300 mm diameter) filled with 3 kg of soil mixture containing red soil, sand and farmyard manure (FYM) at 1:1:1 ratio. All the pots were watered to the field capacity with tap water up to 19 DAS. On 20 DAS the pots were irrigated with tap water for control, 100 mM NaCl, 5 mM $CaCl_2$ and 100 mM NaCl + 5 mM $CaCl_2$ solutions. The plants were uprooted randomly on the 30 and 50 DAS and used for estimating the pigments and enzyme activities.

Chlorophyll and carotenoid determination

Extraction and determination of chlorophyll and carotenoid was performed according to the method of Arnon (1949). Five hundred milligrams of fresh leaf material was ground with 10 ml of 80% acetone at 4°C and centrifuged at 2500 rpm for 10 minutes at 4°C. This procedure was repeated until the residue became colourless. The extract was transferred to a graduated tube and made up to 10 ml with 80% acetone and assayed immediately. Three milliliters aliquots of the extract were transferred to a cuvette and the absorbance was read at 645, 663 and 480 nm with a spectrophotometer (U-2001-Hitachi) against 80% acetone as blank. Chlorophyll content was calculated using the formula of Arnon and expressed in mg g^{-1} fresh weight (FW).

Total chlorophyll (mg/ml)= $(0.0202) \times (A.645) + (0.00802) \times (A.663)$

Chlorophyll 'a' (mg/ml)= $(0.0127) \times (A.663) - (0.00269) \times (A.645)$

Chlorophyll 'b' (mg/ml)= $(0.0229) \times (A.645) - (0.00468) \times (A.663)$

Carotenoid content was estimated using the formula of Kirk and Allen (1965) and expressed in mg g^{-1} FW.

Carotenoid = $A.480 + (0.114 \times A.663 - 0.638 \times A.645)$.

Assay of γ-Glutamyl kinase activity

γ-Glutamyl kinase activity was assayed by the method of Hayzer and Leisinger (1980). Plant sample (1 g) were extracted with 50 mM Tris-HCl buffer. Centrifugation at 40,000 g for 30 minutes at 4°C. 0.1 ml reaction buffer adding 0.1 ml 10 × ATP and 1.8 ml of extract. Incubate 37°C for 30 minutes add 2 ml of stop buffer was added. γ-Glutamyl kinase activity measured by a spectrophotometer at 535 nm and expressed in units (U). One unit of is defined as μg of γ-glutamylhydroxamate formed min^{-1} mg^{-1} protein.

Assay of Proline oxidase activity

Proline oxidase activity was determined according to the method outlined by Huang and Cavalieri (1979). Plant samples (1 g) were extracted with 5 ml of Tris-HCl buffer pH 8.5 grinding medium. Centrifuge at 10,000 g for 10 minutes at 4°C. Take the supernatant again centrifuge at 25,000 g at 20 minutes at 4°C. Take 3 ml assay mixture containing 0.1 ml of extract. Add 1.2 ml of 50 mM Tris HCl buffer pH 8.5, 1.2 ml of 5 mM $MgCl_2$, 0.1 ml of 0.5 mM NADP, 0.1 ml of 1 mM KCN, 0.1 ml of 1 MM phenazine methosulphate (PMS), 0.1 ml of 0.06 mM 2,6-dichlorophenol indophenol (DCPIP), 0.1 ml distilled water instead of proline, the reaction was monitored at 600 nm at 25°C using proline to initiate reaction, the OD value increased was noted

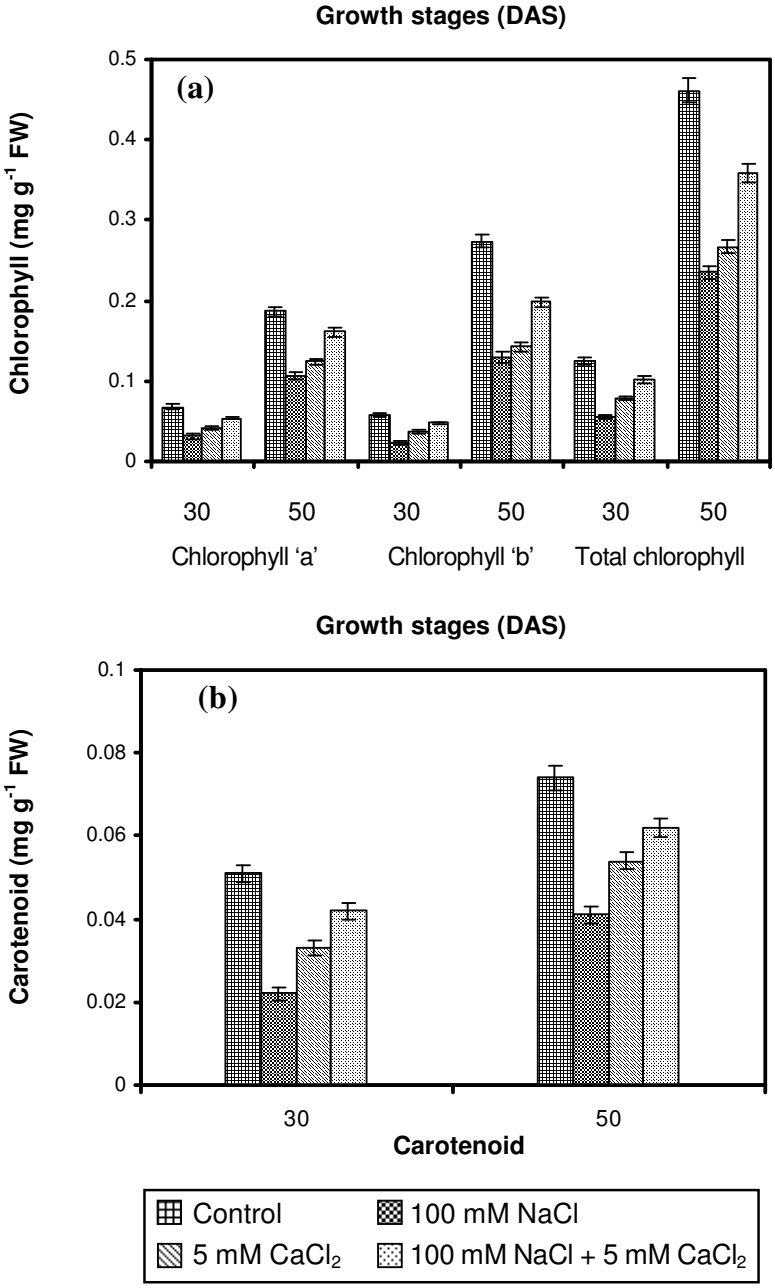

Fig 1. Interactive effects of NaCl and $CaCl_2$ on (a) chlorophyll and (b) carotenoid contents (mg g^{-1} FW) of *Withania somnifera*. The data are $\overline{X} \pm$ SD of 3 replicates.

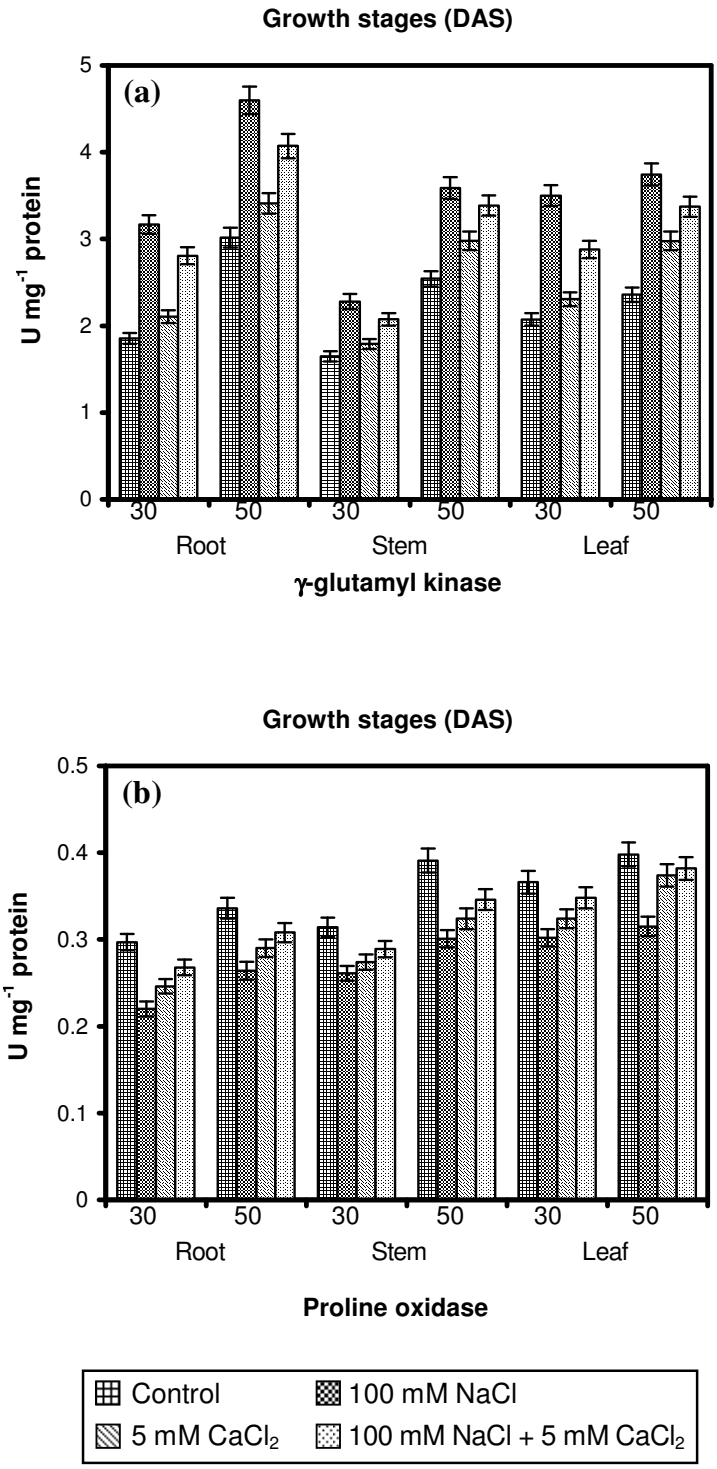

Fig 2. Interactive effects of NaCl and $CaCl_2$ on (a) γ-glutamyl kinase, (b) proline oxidase activities (U mg^{-1} protein) of *Withania somnifera*. The data are $\overline{X} \pm$ SD of 3 replicates.

0, 1, 2, 3, 4 and 5 min. Proline oxidase activity was expressed in U (one U = mM DCPIP reduced min^{-1} mg^{-1} protein).

Statistical analaysis

Each treatment was analysed with at least three replicates and a standard deviation (SD) was calculated and data are expressed in $\overline{X} \pm$ SD of three replicates.

Results and discussion

Chlorophyll and carotenoid contents

The chlorophyll content increased with the age in leaves of control and treated plants, however, a reduction in chlorophyll 'a', 'b', total chlorophyll and carotenoid contents have been observed in the NaCl stressed *Withania somnifera* plants when compared to control (Figs 1a and 1b). The reduction in leaf chlorophyll under salinity has been attributed to the destruction of the chlorophyll pigments and the instability of the pigment protein complex (Levitt 1980). It is also attributed to the interference of salt ions with the denovo synthesis of proteins, the structural component of chloroplast rather than the break down of chlorophyll (Megdiche et al., 2008). The NaCl with $CaCl_2$ increased the chlorophyll content when compared to NaCl stressed plants.

γglutamyl kinase activity

The γ-glutamyl kinase activity has been increased in root, stem and leaf to a large extent in the NaCl; $CaCl_2$ stressed *Withania somnifera* plants when compared with control (Fig 2a). NaCl with $CaCl_2$ treated plant decreased in γ-glutamyl kinase activity when compared to NaCl stressed plants. The induction of proline accumulation may be due to an activation of proline synthesis through glutamate pathway involving γ-glutamyl kinase, glutamyl phosphate reductase and Δ^1-pyroline-5-carboxylate reductase activities (Jaleel et al., 2008e,f). The other enzymes involved in the proline biosynthetic pathway i.e., proline-5-carboxylate reductase has reported to increase under stress (Delauney and Varma, 1990; Jaleel et al., 2007h).

Proline oxidase activity

Proline oxidase activity has been inhibited by the NaCl, $CaCl_2$ stress to a large extent in all parts of *Withania somnifera* when compared with control (Fig 2b). Addition of NaCl with $CaCl_2$ treated plant increased the proline oxidase activity when compared to NaCl stressed plants. Proline oxidase, oxidise the proline and convert it back to glutamic acid. This enzyme also influences the level of free proline. Proline accumulation in salt stressed cell may also be explained by a decreased oxidation of proline during salt stress as reported in *Catharanthus* (Jaleel et al., 2007d). The activity of proline degrading enzymes, proline oxidase and proline dehydrogenase were significantly inhibited in the salt stressed *Dioscorea rotundata* (Jaleel et al., 2008e). The decrease in proline oxidase activity with concomitant increase in γ-glutamyl kinase activity might be the reason for higher proline accumulation in NaCl stressed *Withania somnifera* plants. From these results, it can be concluded that the addition of $CaCl_2$ to NaCl stressed plants have a significant role in partial alleviation of salinity stress.

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