Original Article

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NaCl as a physiological modulator of synthesis of compatible solutes and antioxidant potential in sangam (*Clerodendron inerme* L.)

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ABSTRACT

The present investigation was made to study the effect of different concentrations of sodium chloride on the proline (PRO), glycine betaine (GB), sugar and antioxidant content of the halophytic species, *Clerodendron inerme*. The plant could survive a wide range of 100-1000 mM of NaCl. The upper limit for the survival of the species was 500 mM. Above 500 mM, the seedlings could not survived. However, favorable growth response by the seedlings was confined to 200 mM NaCl. The accumulation of PRO, GB was more in leaf tissue than the stem and root of NaCl treated plants. PRO, GB content is believed to function as a compatible solute in balancing cytoplasmic and vacuolar water potentials not only due to salinity stress but also under drought, heat and cold stresses. Increasing salinity increased the level of PRO, GB up to the extreme level of 500 mM NaCl. The sugar content decreased in all the three tissues with increasing NaCl up to 200 mM and at higher concentrations, salinity gradually increased the sugar up to 500 mM NaCl. Survival of plants in the saline environment depends on the quantitative ratio between toxic and protective compounds PRO; GB is believed to be one of the protective substances. The non-enzymatic antioxidant contents such as ascorbic acid and α-tocopherol were detected under high concentration of 500 mM NaCl. The increase in antioxidant enzyme activity could be the response of cellular damage induced by the NaCl.

KEY WORDS: Antioxidant, glycine betaine, halophytes, proline, salinity, sugar

INTRODUCTION

Soil salinization is one of the most severe plant growth, agricultural yield and finally ecological problems (Hasegawa, 2013). Al-Sadi *et al*. (2010) reported that just about 400 million per hectare of land is affected as a result of salinity. Soil salt affects plant growth mainly by the action of some ions, such as Na^+ and Cl^- (Jamil *et al*., 2012). Ions taken up by roots not only accumulate at high concentrations in plant tissues, but may also reduce the uptake of other ions, like nutrient elements (Dong, 2012; Radhakrishnan and Kumari, 2012). Salt tolerance can be achieved by salt exclusion or salt inclusion, specifically, salt excluders exhibit water deficit that reduces plant growth and requires a mechanism for prevention of and internal water deficit. In this sense, its hypothetical salt tolerance by salt inclusion requires either high tissue tolerance to Na^+ and Cl^- or avoidance of high tissue concentration (Patel *et al*., 2010).

Three most important factors contribute to salinization in agricultural areas: (i) poor irrigation management and lack of suitable drainage; (ii) irrigation with saline water; and (iii) rising groundwater tables because of vegetation changes (Rewald *et al*., 2012). Salinity causes numerous challenges for plants, including water stress, mal-nutrition and accumulation of excess ions to toxic levels (Rewald *et al*., 2011). Plants are subjected to a number of environmental stresses that harmfully affect plant growth, metabolism and biological yield (Lawlor and Cornic, 2002, Kheybari *et al*., 2013). The environmental stresses such as drought, air pollution, temperature, salinity, heavy metals, pesticides, and soil pH are major factors limiting agricultural crop production because, they affect almost all plant functions (Hern-Ndez *et al.,* 2001;Yue *et al*., 2011).

Plants have both enzymatic and non-enzymatic mechanisms for scavenging reactive oxygen species. The enzymatic antioxidants include superoxide dismutase, catalase, guaiacol

peroxidase, and the enzymes of ascorbate glutathione (AsA-GSH) cycle such as AsA peroxidase, dehydro AsA reductase (DHAR), mono DHAR, and GSH reductase. AsA, GSH, phenolics, carotenoids, and tocopherols, which act as potent non-enzymic antioxidant inside the cell (Sharma *et al*., 2012). All reactive oxygen species are extremely harmful to organisms at high concentrations. When the level of reactive oxygen species is higher than the tolerance level, a cell is subjected to "oxidative stress." The improved production of reactive oxygen species during environmental stresses can adversely affect the cellular activities by causing the oxidation of proteins, peroxidation of lipids, and preventing the activity of enzymes, which eventually results in cellular deactivation (Sharma *et al*., 2012).

Halophytes are remarkable plants able to tolerate salt concentrations that kill 99% of other species (Flower and Colmer, 2008). Halophytes show growth, development and survival under saline conditions and the effect of salinity on growth varies among species (Flower and Colmer, 2008) in addition to genotypes or clones and, based upon the tolerance capacity of plants, halophytes are broadly categorized into "salt accumulator" and "salt excluder." Salinity stresses both categories of plant in two ways, high concentrations of salts in the soil make it harder for the roots to extract water and create toxicity to the cytoplasm within the plant (Munns and Tester, 2008; Lokhande and Suprasanna, 2012). Osmotic adjustment under saline conditions can occur in plants by the uptake of inorganic ions from the medium, compartmentalizing ions in the cell vacuole and balancing osmotic potential in vacuoles by the synthesis of compatible organic solutes in the cytoplasm (Ashraf and Harris, 2004; Abdel Latef *et al*., 2009).

Above one mechanism is operating in salt tolerant plants against salinity, yet it is therefore important to study the mechanism operating at each level in full detail so as to develop a complete understanding of salt stress and utilizing this knowledge improvement of crops against salt stress. Halophytes can be very helpful under such situations they can be used for industrial, ecological and agricultural purposes (Koyro *et al*., 2011).

The objective of the present investigation was to study the effects of salinity stress on compatible solutes and antioxidant enzymes of *Clerodendron inerme*.

MATERIALS AND METHODS

Plant Material

The mature stem cuttings were collected from salt marshes in the mangrove area of Pichavaram, on the east coast of Tamil Nadu, India about 10 km East of Annamalai University Campus.

Growth Conditions

The stem cuttings of *C. inerme* (3 cm long with one node and two opposite leaves) were planted individually in polythene bags (7"×5") filled with homogenous mixture of garden soil containing red earth, sand and farm yard manure (1:2:1). The cuttings were irrigated with tap water and maintained in the botanical garden, Annamalai University.

Salt Treatment and Experimental Design

One-month-old and well-established cuttings were selected and treated with varying concentrations of NaCl (100-1000 mM). The stem cutting grown above 500 mM NaCl concentrations did not survive after 10 days of treatment, the experimental plant treated with NaCl up to 500 mM were alone maintained in the experimental site. The experimental yard was roofed with transparent polythene sheet at a height of 3 m from the ground in order to protect the plants from the rain.

Samples were collected randomly on 60th; 90th and 120th day after treatment. The seedlings were separated into leaves, stem and root and used for analyses.

Determination of Compatible Solutes

Determination of proline (PRO) content

PRO was extracted and estimated by following the method of Bates *et al*. (1973).

Extraction

Five hundred milligram of fresh plant material was homogenized in a mortar and pestle with 10 ml of 3% aqueous sulfosalicylic acid. Then the homogenate was filtered through Whatman No. 1 filter paper. The residue was re-extracted and pooled, and the filtrate was made up to 20 ml with aqueous sulfosalicylic acid, and this extract was used for the estimation of PRO.

Estimation

To 2 ml of PRO extract, 2 ml of acid ninhydrin and 2 ml of glacial acetic acid were added. The mixture was incubated for an hour at 100°C in a boiling water bath. Then the test tubes containing mixture were transferred to an ice bath to terminate the reaction. Then 4 ml of toluene was added and mixed vigorously using a test tube stirrer for 20 s and the toluene containing the chromophore was separated from the aqueous phase with the help of a separating funnel and the absorbance was measured at

520 nm in a spectrophotometer using a reagent blank. The PRO content was determined from a standard curve with PRO, and the results were expressed in milligram per gram dry weight.

Preparation reagent

Acid-ninhydrin reagent

To 1.25 g of ninhydrin, 30 ml warm glacial acetic acid, 20 ml of 6 M phosphoric acid were added with agitation.

Determination of glycine betaine (GB) content

GB activity was assayed by the method of (Grieve and Grattan, 1983).

Extraction

Five hundred mg of finely ground dried plant samples was mechanically shaken with 20 ml of de-ionized water for 24 h at 25°C. The time required for this step was determined by extracting the plant samples for 1, 4, 16, 24 and 48 h. The samples were then filtered, and filtrates were stored in the freezer for analysis.

Estimation

Thawed extracts were diluted with 2N H2SO4 (1:1). The acid potassium triiodide solution for total QACs were prepared by dissolving 7.5 g resublimed iodine and 10 g potassium iodide in 1 M HCI and filtered (Speed and Richardson, 1968). Precisely, 0.2 ml of acid potassium triiodide reagent was added to an aliquot of a sample containing between 10 and 15 µg of QACs in water. The mixture was shaken and left for at least 90 min in an ice bath with intermittent shaking. Two ml of ice-cold water was added rapidly to the mixture to reduce the absorbance of the blank and to improve replication. This was quickly followed by 10 ml of 1, 2-dichloroethene in ice, and the 2 layers mixed well and kept at 4°C (Storey and Wyn Jones, 1977). The absorbance of the lower organic layer was measured at 365 nm in a spectrophotometer. The results are expressed as GB equivalent by using GB for standard value.

Determination of total soluble sugar content

Soluble sugars (reducing and non-reducing) were estimated by the modified method of Nelson (1944).

Extraction

Non-reducing sugars were hydrolyzed to reducing sugar, and total sugars were estimated.

Hydrolysis

One ml of the extract was evaporated to dryness in a boiling water bath. To the residue, 1 ml of distilled water and 1 ml of 6 N sulphuric acid were added. The mixture was hydrolyzed by incubating in a water bath at 50°C for an hour. The solution was neutralized with 1 N sodium hydroxide and made up to 10 ml with distilled water and used for the estimation of total sugars.

Estimation

A volume of 1 ml fresh copper reagent and 1 ml of an extract (prepared by mixing copper tartrate solution and copper sulphate solution [25:1 v/v]) were added. The mixture was heated in a Folin-Wu-tube with its mouth covered with a marble in a boiling water bath for 20 min, then cooled and 1 ml of arsenomolybdate reagent was added. The final volume was made up to 20 ml with distilled water. The resultant blue color was read at 520 nm in a spectrophotometer against the appropriate blank. The sugar content was expressed in milligram per gram dry weight. The content of the sugar was calculated from the standard graph prepared with glucose.

Reagent - copper tartrate solution

To 25 g of sodium carbonate (anhydrous), 25 g of sodium potassium tartrate, 20 g of sodium bicarbonate and 200 g of sodium sulfate (anhydrous) were dissolved in 800 ml distilled water, filtered and made up to 1000 ml with distilled water and stored in a brown bottle at room temperature.

Copper sulfate solution

To 15 g of copper sulfate and two drops of concentrated sulphuric acid were added to 100 ml of distilled water.

Arsenomolybdate reagent

In 450 ml of distilled water, 25 g of ammonium molybdate, 21 ml of concentrated sulphuric acid and 3 g of sodium arsenate dissolved in 25 ml of distilled water were added and the mixture was kept in an incubator at 37°C for 48 h and filtered. The reagent was stored in a brown bottle at room temperature.

Determination of Enzymes

Determination of ascorbic acid (AA) content

AA was extracted and estimated by the method of Omaye *et al*. (1979).

Extraction

One gram of plant tissue was homogenized in a pestle and mortar with 5 ml of 10% trichloroacetic acid (TCA) and centrifuged at 3500 g for 20 min. The pellet was re-extracted twice with 10%TCA and supernatant was made to 10 ml and used as an extract.

Estimation

To one ml of dinitrophenylhydrazine; thiourea and copper sulfate reagents were added to 0.5 ml of extract and

mixed thoroughly. Then the tube was incubated at 37°C for 3 h and to this 0.75 ml of ice-cold 65% sulphuric acid was added. The tubes were then allowed to stand at 30°C for 30 min. The resulting color was read at 520 nm in a spectrophotometer (U-2001–Hitachi). The AA content was determined using a standard curve prepared with AA and the results were expressed in mg per gram dry weight.

Preparation of reagent

DTC reagent

To 3 g of 2, 4-dinitrophenylhydrazine (DNPH), 0.4 g of thiourea and 0.05 g of copper sulfate were added and dissolved in 100 ml of 9 N sulphuric acid. Standard solution 10 mg/100 ml 10% TCA.

Determination of α-tocopherol (α-toc)

α-Tocopherol activity was assayed as described by Backer *et al.* (1980).

Extraction

Five hundred milligram of fresh tissue was homogenized with 10 ml of a mixture of petroleum ether and ethanol (2:1.6 v/v) and the extract was centrifuged at 10,000 rpm for 20 min and the supernatant was used for the estimation of α-tocopherol.

Estimation

To 1 ml of extract, 0.2 ml of 2% 2, 2-dipyridyl in ethanol was added and mixed thoroughly and kept in dark for 5 min. The resulting red color was diluted with 4 ml of distilled water and mixed well. The resulting color in the aqueous layer was measured at 520 nm. The α-tocopherol content was calculated using a standard graph made with the known amount of α-tocopherol.

Statistical Analysis

Data were analyzed for significance using one-way analysis of variance and the differences contrasted using a Duncan's multiple range test at $P \leq 0.05$. All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS, version 16).

RESULTS

The PRO, GB content in the three different plant tissues increased with increase in NaCl concentrations up to the extreme level on all the sampling days (Figures 1 and 2), respectively. Total sugar content decreased in the leaf, stem and root with increase in NaCl up to optimum level and at higher concentrations, salinity gradually increased the total sugar content up to the extreme level of NaCl concentration when compared to control (Figure 3). The leaf had more PRO, GB and total sugar when compared to stem and root in all the sampling days. There was a considerable increase in the AA and α-tocopherol content of leaves up to the extreme level of NaCl concentration when compared to control (Figures 4 and 5), respectively.

DISCUSSION

Compatible solutes play a role in plant osmotolerance by different ways, protecting enzymes from denaturation, stabilizing membrane or macromolecules or playing adaptive roles in mediating osmotic adjustment (Ashraf and Foolad, 2007). The function of the compatible solutes is not limited to osmotic balance. Compatible solutes are typically hydrophilic, and possibly capable to replace water at the surface of proteins or membranes, thus acting as low molecular weight chaperones (Hasegawa *et al.*, 2000). These

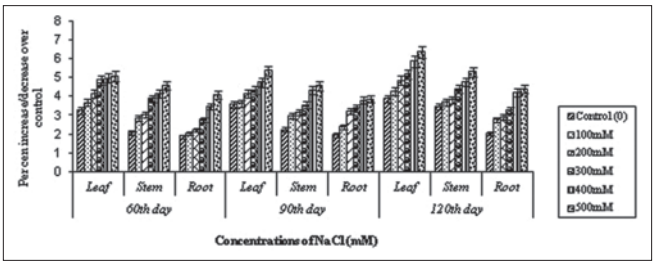

Figure 1: Accumulations of proline (PRO) content in *Clerodendron inerme* under different concentrations of NaCl stress after 60th, 90th and 120th days. Values are given as mean ± SD of five replicates

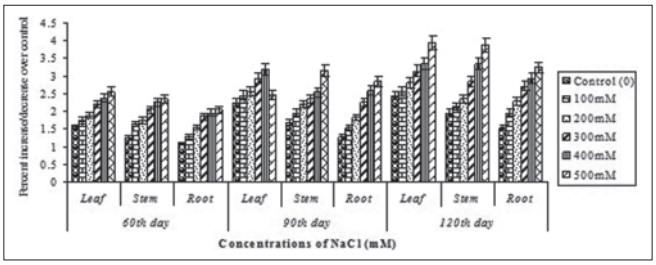

Figure 2: Accumulations of glycine betaine (GB) content in *Clerodendron inerme* under different concentrations of NaCl stress after 60th, 90th and 120th days. Values are given as mean ± SD of five replicates

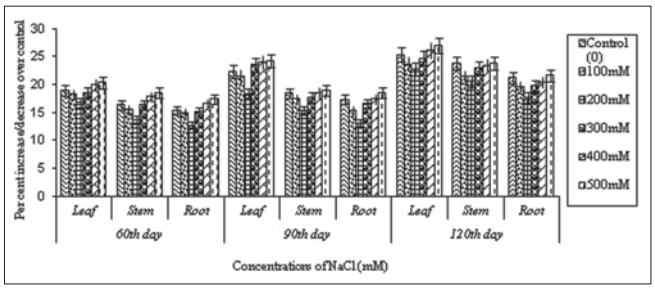

Figure 3: Accumulations of sugar content in *Clerodendron inerme* under different concentrations of NaCl stress after 60th, 90th and 120th days. Values are given as mean ± SD of five replicates

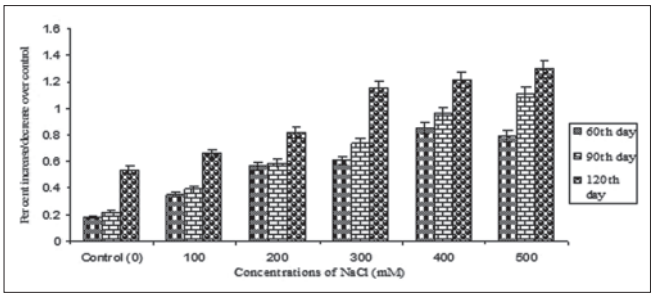

Figure 4: Effect of NaCl on ascorbic acids content of leaves of *Clerodendron inerme* on 60th, 90th and 120th day after the treatment

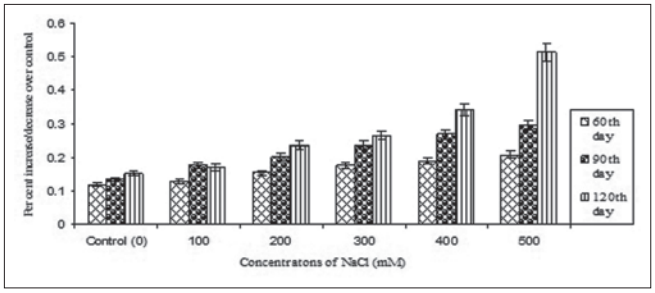

Figure 5: Effect of NaCl on α-tocopherol contentof leaves of *Clerodendron inerme* on 60th, 90th and 120th day after the treatment

solutes also function to protect cellular structures through scavenging reactive oxygen species (ROS) (Hasegawa *et al.*, 2000; Zhu, 2001). Compatible solutes are small molecules, water soluble and uniformly neutral with respect to the perturbation of cellular functions, even when present at high concentrations (Sakamoto and Murata, 2002; Yancey *et al.*, 1982). They comprise nitrogen-containing compounds such as amino acids, amines and betaines, but also organic acids, sugars and polyols (Mansour, 2000).

The accumulation of PRO was more in the leaf tissues than in the stem and root tissues of NaCl treated plants. The higher accumulation of PRO was observed at 500 mM concentration. Recent studies indicate that adaptation to salinity is closely associated with PRO accumulation. A significant increase in PRO content was found only at high salinity (Wang *et al.*, 2006). This is consistent with finding reported on *Suaeda physophora* and *Haloxylon persicum* (Song *et al.*, 2006) and *Sorghum bicolor* (Lacerda *et al.*, 2003). Compatible solutes appear to have additional functions during the stress response acting as "osmoprotectants" either by direct stabilization of protein and membrane structures under dehydration conditions or by protecting the cell against oxidative stress as scavengers of reactive oxygen species (Zhu 2001; Maggio *et al.*, 2002; Marcum, 2006).

PRO accumulation normally occurs in the cytosol where it contributes substantially to the cytoplasmic osmotic adjustment (Ketchum *et al.*, 1991; Turanl *et al.*, 2009; Thippeswamy *et al.*, 2010). Generally salt stress induces PRO accumulation in many halophytes (Brown *et al.*, 2006; Koyro, 2006; Song *et al.*, 2006). The present observations are in accordance with several studies that PRO content progressively increased with high levels of salinity in *Thellungiella halophila* (Inan *et al.*, 2004); *Sesuvium* (Ramani *et al.*, 2006) and *Odyssea paucinervis* (Naidoo *et al.*, 2008). Our result is supporting the findings in barley (Sadeghi, 2009), *Morus alba* (Kumar *et al.*, 2003), (Ahmad *et al.*, 2007), wheat (Karmous *et al.*, 2013), rapeseed (Farhoudi, 2011) and pepper (Chookhampaeng, 2011) where salt stress resulted in extensive PRO accumulation.

GB, an amphoteric quaternary amine plays an important role as a compatible solute in plants under various stresses particularly low temperature and drought (Sakamoto and Murata, 2002). The molecular features of GB allow it to interact with macromolecules, stabilizing the structures and activities of enzymes and protein complexes (Xing and Rajashekar, 2001). GB is a compatible solute, and this suggested that salt probably appears to be concentrated in vacuole and GB accumulated in the cytoplasm (Takemura *et al.*, 2000). Metabolic engineering of GB biosynthesis by the insertion of foreign genes from plants or microbes in plants not naturally accumulating it improved their tolerance to salt, drought and extreme temperature stresses, despite the very low amounts of GB accumulated by these plants (Sakamoto and Murata 2002; Sulpice *et al.*, 2003; Chen and Murata 2008; Ashraf and Akram 2009).

An increase in sugar content and a corresponding decrease in the starch at higher salinities has been reported in several halophytes (Joshi *et al.,* 2002). Singh (2004) proved that a greater accumulation of sugars lowers the osmotic potential of cells and reduces the loss of turgidity in tolerant genotypes. This trend is confirmed in our results which proved a greater increase in soluble sugars content in leaves of coriander with the increase of NaCl concentration. Our finding agrees with researchers done on rice (Siringam *et al.*, 2011), sorghum (Gill *et al.*, 2003), sugar beet (Khavari-Nejad *et al.*, 2008), potato (Farhad *et al.*, 2011) and pistachio (Abbaspour *et al.*, 2012). An increase in sugar and starch content with the increasing NaCl salinity at an optimum level has been reported in *Avicennia officinalis* (Ranganathan *et al.,* 2001).

The antioxidant resistance mechanism may provide a strategy to improve salt tolerance and processes underlying antioxidant responses to salt stress be obliged to be clearly understood. Earlier studies have suggested a pivotal role for subcellular compartmentation in antioxidant defense

mechanism under stress conditions and NaCl stress (Gomez *et al*., 1999). Recently, a correlation between the antioxidant capacity and salt tolerance has been found in different halophytic plant species, including *Centaurea tuzgoluensis* (Yildiztugay *et al*., 2011). *Plantago maritima* (Sekmen *et al*., 2007) and *Cakila maritima* (Amor *et al*., 2006).

AA is the majority abundant, influential and water soluble antioxidant acts to prevent or in minimizing the damage caused by ROS in plants (Smirnoff, 2005; Athar *et al*., 2008). Increased AA contents in *Hordeum vulgare* plants irrigated with saline water has also been recorded by Sarwat and El-Sherif (2007). A 30 per cent increase in AA content in tomato fruits grown under saline conditions has been reported by Kim *et al*. (2008). The increase in AA, when wheat seeds were presoaked with gibberellic acid and salicylic acid under saline conditions, has been reported (Seth *et al*., 2007). Azooz and Al-Fredan (2009) recorded an increased content of endogenous AA under saline conditions when plants were treated with exogenous AA in *Vicia faba*. According to them, AA plays an inductive role in overcoming the detrimental effects of seawater salinity. Similarly, in the leaves *Cicer arietinum* cv. Abrodhi, the AA content has been reported to increase with increasing NaCl concentration (Mishra *et al*., 2009). According to them, AA plays an inductive role in overcoming the detrimental effects of seawater salinity. Khan *et al*. (2010) also reported reduced uptake of sodium in AA treated *Brassica* when grown under saline conditions. In accordance with them, AA can mitigate the harmful effects of salinity when applied as a seed soaking agent.

Sodium chloride treatments increased the α-tocopherol content in *C. inerme*. Tocopherols are considered as a major antioxidant in biomembranes, where they play both antioxidant and non-antioxidant functions. Tocopherols are considered general antioxidants for the protection of membrane stability, including quenching or scavenging ROS like $^1O_2$. Tocopherols are localized in plants in the thylakoid membrane of chloroplasts. Of four isomers of tocopherols (α-, β-, γ-, δ-) found in plants, α-tocopherol has the highest antioxidative activity due to the presence of three methyl groups in its molecular structure (Kamal-Eldin and Appelqvist, 1996). Recently, it has been found that oxidative stress activates the expression of genes responsible for the synthesis of tocopherols in higher plants (Wu *et al*., 2007). Increased levels of α-tocopherol and ASH have been found in tomato following trizole treatment which may help in protecting membranes from oxidative damage and thus chilling tolerance in tomato plants (Shao *et al*., 2007). Increase in tocopherol during water stress in plants has also been reported by many workers (Wu *et al*., 2007; Shao *et al*., 2007). Being the major antioxidant species in plants, the AA, GSH and α-tocopherol contents vary in different subcellular compartments, according to the intensity of stress (Gaspar *et al*., 2002).

CONCLUSION

The present study shows that *C. inerme* is a moderately salt tolerant species. Sodium chloride salinity stimulated its, organic constituents and certain key enzymes up to the extreme concentration of 500 mM NaCl. Hence, it is concluded that this species could be recommended for cultivation in salt affected soils to reduce the soil salinity level.

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