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Morphological and Physio-biochemical Changes of *in Vitro* Cactus (*Echinopsis calochlora*) in Responses to Salt Stress

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Summary

Na^+ content in the plantlets was increased upon exogenous NaCl in the culture medium whereas the potassium ion (K^+) decreased. Free proline content in salt stressed cactus plantlets was also enriched and peaked when plantlets were subjected to 200 mM NaCl. Na^+ in salt stressed plantlets was negatively correlated to chlorophyll a content. Photosynthetic pigments, i.e., chlorophyll a, chlorophyll b, total chlorophyll and total carotenoids, declined significantly in plantlets exposed to 100 mM NaCl, resulting in reduced net photosynthetic rate and inhibition of overall growth characters. Number of cladode budding, root length, number of roots, and fresh and dry weight of cactus plantlets was significantly declined when cactus plantlets expose to 50 mM NaCl. In addition, the number of roots of *in vitro* cactus plantlets was a good indicator for salt sensitivity, which were absent in plantlets subjected to 200 mM NaCl for 28 days.

Key words. Chlorophyll – growth – net photosynthetic rate – proline – root trait – sodium ion

Introduction

Salt affected soil is a major problem world over and is estimated to affect ~20 % of irrigated area in the world. As per FAO, 397 Mha (17 % of cultivated area) has been estimated to be salt-affected (Qadir et al. 2008). Of this, 195 Mha (~49 %) is in Asia Pacific and Australia, 92 Mha (23 %) in Near East, 61 Mha (15 %) in Latin America and 39 Mha (10 %) in Africa. In arid and semiarid zones, salt affected soil and the distribution areas have increased drastically due to both direct salt build-up and secondary salinization effects (Pitman and Läuchli 2002). Salt contamination in the salt affected soil is a key factor that inhibits growth and development in plants, especially glycophyte including most crop species (Parida and Das 2005; Läuchli and Grattan 2007). Halophytes have developed salt defence mechanisms to grow in the inland salt affected soil i.e. ion homeostasis, osmoregulation, antioxidant system and hormonal regulation (Hasegawa et al. 2000). Therefore, the key successes of salinity phytoremediation using halophytic species compose of an edible crop, bio-fuel or biomass, fruit crop, animal feed and quick adaptation to inland environment with high productivity (Flowers and Colmer 2008; Flowers et al. 2010). Cactus, a member of crassulacean acid metabolism (CAM) plant species, is a candidate plant adapted to arid zones, have reduced leaves and sunken stomata to prevent the water loss and uptake CO_2 in the darkness (Lüttge 2004). In addition, CAM plants have long been adapted to photosynthesis coping with abiotic stress, i.e., drought, extreme light intensity, extreme temperature and salt stresses (Lüttge 1993; Cervera et al. 2007). There are many cacti species that grow in arid area as a fruit crop, e.g., columnar cacti (Nerd et al. 1993; Elobeidy 2004). Osmoregulation as plant defense mechanism is one of the most prevalent strategies in higher plants to survive in abiotic stress conditions. Proline is a neutral osmolyte that gets enriched in the cells without toxicity for osmotic potential control. Proline biosynthesis *via* P5CS (Δ^1-pyrroline-5-carboxylate synthase) activity and proline enrichment in salt stressed cactus has already been demonstrated as an osmoprotective agent in the cactus plant exposed to NaCl stress (Silva-Ortega et al. 2008). In addition, two major defense mechanisms in cactus have been identified as 1) ion channel and selective ion transport 2) CO_2 assimilation by CAM (Noble et al. 1984; Osmond et al. 1987; Lüttge 1993).

In the previous reports, the salt stress responses in *Opuntia* cactus species have been investigated in the greenhouse (Cony et al. 2006; Silva-Ortega et al. 2008) and field trials (Nerd et al. 1993; Cavalcante et al. 2007). Up to date, plant biotechnology including plant tissue culture or *in vitro* has been used as a potential tool by research scientists to reduce the environmental effects in

cacti (Shedbalkar et al. 2010). For example, cacti micropropagation has been well established in *Opuntia lanigera* (Estrada-Luna et al. 2008; Angulo-Bejarano and Paredes-López 2011), *Hylocereus costaricensis* (Viñas et al. 2012), *Cereus peruvianus* (Sayad et al. 2004), *Coryphantha elephantidens* (Wakhlu and Bhau 2000), *Mammillaria* spp. (Ramirez-Malagon et al. 2007) and *Turbinicarpus* spp. (Dávila-Figueroa et al. 2005). However, there is no study investigating the salt stress responses of *in vitro* cactus grown under photoautotrophic condition (sugar-free medium), especially in *Echinopsis* species. The aim of the present investigation was to investigate the physiological and growth characteristics of *Echinopsis calochlora* grown under photoautotrophic *in vitro* in responses to salt stress.

Materials and Methods

Plant Materials and Salt Stress Treatment

Cladodes of *Echinopsis calochlora* were surface-sterilized with 70 % (*v/v*) ethanol for 2–3 min followed by 10 % sodium hypoclorite (Chorox®) for 10 min, and then rinsed thrice with sterilized double distilled water. The sterilized materials were proliferated on MS (Murashige and Skoog 1962) medium containing 0.5 mg L^{-1} N^6-benzyl amino purine (BA), 3 % sucrose (*w/v*) and 0.2 % Phytagel® (*w/v*) for 6 weeks. Multiple cladode buddings were individually transferred to hormone-free MS medium for 4 weeks. Cactus plantlets were cultured *in vitro* under 28 ± 2 °C ambient temperature, 60 ± 5 % relative humidity (RH) and 16 h d^{-1} photoperiod of 65 ± 5 μmol m^{-2} s^{-1} of photosynthetic photon flux density (PPFD) provided by fluorescent lamps. The uniform cladode shoots (1.5 ± 0.1 cm in height) were excised as initial materials to grow on MS liquid media using vermiculite as supporting material for 4 weeks. The number of air exchange in the culture vessel was adjusted to 2.32 μmol CO_2 h^{-1} by punching a hole over the plastic cap and covering by microporous filter (0.2 μm of pore size, Nihon Millipore Ltd., Tokyo, Japan). The glass vessels containing plantlets were subsequently incubated in a plant growth chamber (Model FLI 2000, ELYELA, Tokyo, Japan) under ambient day/night temperature of 30 ± 2/28 ± 2 °C, 60 ± 5 % RH and 16 h d^{-1} photoperiod of 120 ± 5 μmol m^{-2} s^{-1} PPFD provided by fluorescent lamps, and CO_2 enrichment at 1,000 ± 100 μmol mol^{-1}. Sodium chloride (NaCl) concentration in the culture medium was adjusted to 0 (control), 50, 100 and 200 mM for 28 days. Thereafter, sodium ion, potassium ion, free proline content, photosynthetic pigments, net photosynthetic rate, and growth characters in cactus plantlets were measured.

Assay of Na^+ and K^+

Briefly, one hundred milligram of plant material was ground in liquid nitrogen. Na^+ and K^+ in powdered material were extracted in an acid mixture (HNO_3 and $HClO_4$) and their amount determined using an atomic absorption spectrophotometer (AA, Model M6, Thermo Elemental, MA, USA) as per the method of Dionisio-Sese and Tobita (1998).

Free Proline Content

Free proline in the cladode plantlets was extracted and analyzed according to the method of Bates et al. (1973). Fifty milligrams of fresh material was ground with liquid nitrogen in a mortar. The homogenate powder was mixed with 1 mL of aqueous sulfosalicylic acid (3 %, *w/v*) and filtered through Whatman #1 filter paper. The extracted solution was reacted with an equal volume of glacial acetic acid and ninhydrin reagent (1.25 mg ninhydrin in 30 mL glacial acetic acid and 20 mL 6 M H_3PO_4) and incubated at 95 °C for 1 h. The reaction was terminated by placing the container in an ice bath. The reaction mixture was mixed vigorously with 2 mL toluene. After cooling to 25 °C, the chromophore was measured at 520 nm in a spectrophotometer (HACH DR/4000; Model 48000, HACH Company, Loveland, Colorado, USA) using L-proline as a standard.

Photosynthetic Pigments

Concentrations of chlorophyll a (Chl_a), chlorophyll b (Chl_b) and total chlorophyll (TC) were determined following the method of Shabala et al. (1998) and total carotenoids (C_{x+c}) concentration was measured according to Lichtenthaler (1987). One hundred milligrams of material collected from cladode plantlets were placed in a 25 mL glass vial (Opticlear® KIMBLE, Vineland, New Jersey, USA) and 10 mL of 95.5 % acetone was added to it. The mixture was blended with a homogenizer (T25 basic ULTRA-TURRAX®, IKA, Kuala Lumpur, Malaysia). The glass vials were sealed with Parafilm® to prevent evaporation and then stored at 4 °C for 48 h. The Chl_a, Chl_b and C_{x+c} concentrations were measured using a UV-visible spectrophotometer (DR/4000, HACH, Loveland, Colorado, USA). A solution of 95.5 % acetone was used as blank.

Net Photosynthetic Rate

Net photosynthetic rate (P_n; μmol g^{-1} DW s^{-1}) was calculated by comparing the different concentrations of CO_2 inside (C_{in}) and outside (C_{out}) the glass vessel containing the cactus plantlets. The CO_2 concentrations at steady state were measured in the darkness by Gas Chromatography (GC; Model GC-17A, Shimadzu Co. Ltd., Tokyo, Japan). The P_n of *in vitro* cultivated plantlets was calculated according to the method of Fujiwara et al. (1987).

Growth Parameters

Cladode height, number of cladode budding, root length, number of roots, and fresh and dry weight of cactus

plantlets were measured 28-days after exposure to NaCl stress. Plantlets were dried at 80 °C in a hot-air oven for 48 h, and then incubated in desiccators before the measurement of dry weight.

Experiment Design and Statistical Analysis

The experiment was arranged as completely randomized design (CRD) with 8 replications (n = 8). The mean values obtained were compared using Tukey's HSD and analyzed with SPSS software ver. 11.5.

Results and Discussion

Morphological Characteristics

Morphological characters including plant size, the green colour and the number of cladode budding in cactus plantlets decreased in response to exogenous NaCl in the culture medium, especially at 200 mM NaCl (Fig. 1). Plant height of 100 mM salt stressed plantlets declined by 23 % over the control. The number of cladode budding, root length, number of roots, fresh weight and dry weight declined by 31.76 %, 45.28 %, 41.54 %, 20.65 % and 43.66 %, respectively, over the control, when plantlets were exposed to 50 mM NaCl (Table 1). At very high NaCl stress (200 mM), overall growth performances of salt stressed plantlets dropped sharply, and there was no root initiation. In addition, the basal zone of explants turned brown under 200 mM NaCl salt stress (Fig. 1D).

In general, overall growth performance, i.e., cladode height, number of cladode budding, root length, root number, fresh weight and dry weight, in salt stressed cactus was inhibited. These observations are paralleled by earlier reports highlighting reduction in plant height and biomass of two cactus species, Apple cactus (*Cereus repandus*) and Pitaya agria (*Stenocereus gummosus*), under salt contamination (100–300 mg L^{-1}) (Nerd et al. 1993). Likewise, a decrease in number of cladodes, fresh weight and dry weight in two *Opuntia* spp. was observed under salt stress (Cony et al. 2006). In Pitaya (*Hylocereus undatus*), survival percentage, plant height, root length and plant dry weight declined when subjected to 40 mM NaCl for 75–90 days (Cavalcante et al. 2007). Additionally, the root trait has been reported as salt sensitivity marker in two species of *Opuntia*, i.e. *O. streptacantha* (Silva-Ortega et al. 2008) and *O. ficus-indica* (Murillo-Amador et al. 2001), and pitaya (Cavalcante et al. 2007). Previously, brown coloration has been observed in the basal zone of cladode suggesting toxic effects due to salt stress (350 mM NaCl) (Silva-Ortega et al. 2008).

Na^+, K^+ and Free Proline Contents

In salt stressed plantlets of *in vitro* grown cactus, a significant accumulation of Na^+ was observed, whereas K^+ declined (Fig. 2A). In contrast, Na^+ accumulation in salt

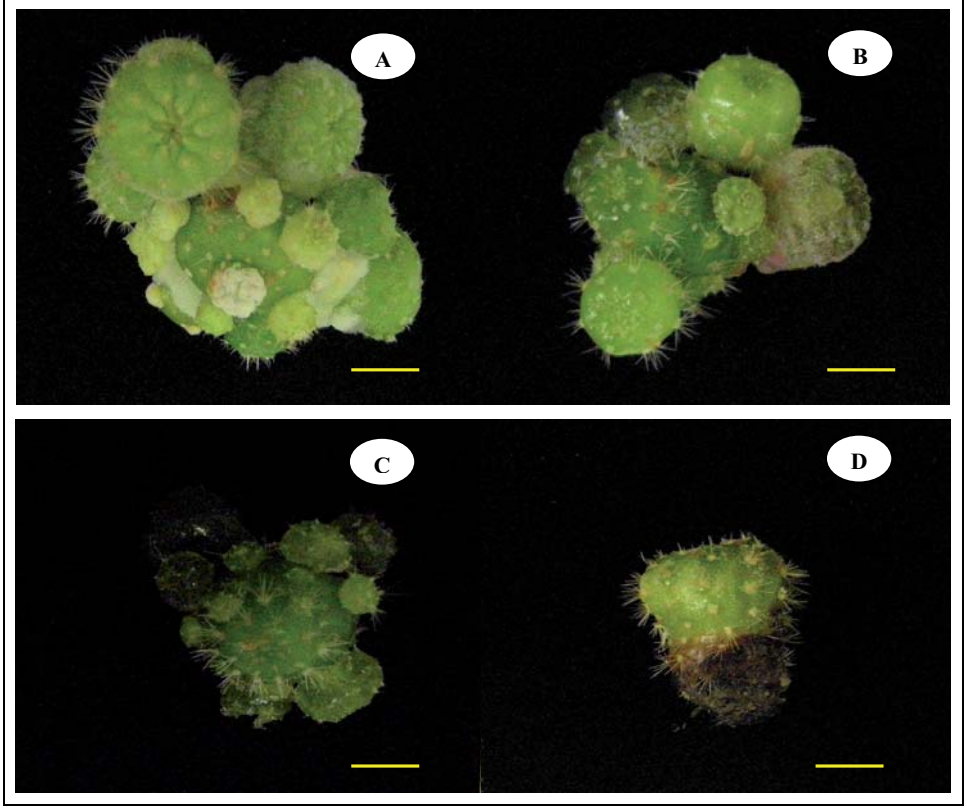


Fig. 1. Morphological characteristics of *Echinopsis* cactus grown *in vitro* under 0 (A), 50 (B), 100 (C) and 200 mM NaCl (D) for 28 days. Scale bars are 2 cm.

Table 1. Plant height (PH), number of cladode budding (NB), root length (RL), number of roots (RN), fresh weight (FW) and dry weight (DW) in *Echinopsis* cactus grown *in vitro* under 0, 50, 100 and 200 mM NaCl for 28 days. Data presented as mean ± SE.

NaCl (mM)	PH (cm)	NB	RL (cm)	RN	FW (g)	DW (g)
0	3.29 ± 0.06 a	8.5 ± 0.3 a	5.3 ± 0.4 a	6.5 ± 0.3 a	18.5 ± 0.7 a	0.71 ± 0.03 a
50	2.93 ± 0.08 a	5.8 ± 0.5 b	2.9 ± 0.1 b	3.8 ± 0.3 b	13.2 ± 0.9 b	0.40 ± 0.04 b
100	2.53 ± 0.05 b	3.3 ± 0.3 c	1.6 ± 0.1 c	1.5 ± 0.3 c	4.9 ± 0.5 c	0.25 ± 0.02 c
200	2.06 ± 0.08 c	1.5 ± 0.3 c	0.0 ± 0.0 d	0.0 ± 0.0 d	2.3 ± 0.1 c	0.16 ± 0.01 c

Different letters in each column show significant difference at $P \leq 0.01$ by the Tukey's HSD.

stressed plantlets was negatively correlated with chlorophyll a (Chl_a) content (Fig. 3A). Parallel to Na^+ content, free proline content enhanced significantly in salt stressed plantlets and peaked to 26 μmol g^{-1} FW under extreme salt stress (200 mM NaCl) for 28 days (Fig. 2B).

Previously, Murashige and Skoog (MS) Fe-EDTA and Heller microelement supplemented with 20 g L^{-1} sucrose, 7 g agar and pH 5.8 has been reported as optimized culture medium for *in vitro* culture of *Echinopsis* species, a model plant of this investigation, has already been reported (Vidican et al. 2009; Vidican and Cachiță-Cosma 2010). In the present study, the MS media was suitable to grow the *Echinopsis calochlora* in both proliferation and root induction stages. Na^+ ion accumulation observed in the present study in the salt stressed cactus was a general phenomenon when plants are subjected to exogenous salt treatment. For example, Na^+ accumulation in three cactus species, i.e. *Echinocactus grusonii*, *Fouquieria splendens* and *Carnegiea gingantea*, depends on the salt concentration (0.6–15 dS m^{-1}) in the soil solution (Schuch and Kelly 2008). In prickly pear (*Opuntia ficus-indica*), Na^+ content in the salt stressed root tissues was enriched relating to exogenous salt concentrations (2–21 dS m^{-1} and 50–200 mM NaCl) (Nerd et al. 1991; Murillo-Amador et al. 2001). In Key tree cactus (*Pilosocereus robinii*), there was higher accumulation of Na^+ when subjected to salt treatment, especially in cv. 'Materna 2', which is highly sensitive to salt stress than in cv. 'Materna 1' (Goodman et al. 2012). In addition, the Na^+ content in cactus species varies with the salinity levels in the field. For example, in Apple cactus (*Cereus peruvianus*) and Pitaya agria (*Stenocereus gummosus*) grown in different salinity field sites of Negev desert, Israel, Besor (EC = 0.9–1.1 dS m^{-1}; 100–300 mg L^{-1} Na^+), Qetura (EC = 3.5–4.5 dS m^{-1}; 240–304 mg L^{-1} Na^+) and Neot Hakikar (EC = 3.7–4.5 dS m^{-1}; 520–1080 mg L^{-1} Na^+) was in the order: Neot Hakikar > Qetura > Besor in both genotypes (Nerd et al. 1993). In the present study, K^+ in salt stressed cactus declined significantly in relation to degree of salt stress. It is in agreement to earlier observation made in salt stressed plants of *Stenocereus gummosus* (Noble et al. 1984; Nerd et al. 1993). In contrast, K^+ in the root tissues of salt stressed pear cactus was enhanced in relation to Na^+ accumulation (Nerd et al. 1991).

Free proline is an osmolyte that is enriched when plants are exposed to abiotic stresses, including salt stress. The observed enrichment in free proline in the *Echinops* is corroborated by earlier findings in cactus pear (*Opuntia streptacantha*), where it peaked to 0.6 μmol g^{-1} DW when plants were exposed to 350 mM NaCl for 11 days (Silva-Ortega et al. 2008), Moreover, the expression level of *OsP5CS* gene, a key gene encoded Δ^1-pyrroline-5-carboxylate synthase (P5CS) enzyme for proline biosynthesis, was increased in cactus pear plants exposed to 350 mM NaCl for 9 days (Silva-Ortega et al. 2008).

Photosynthetic Abilities

Chl_a content in the cactus plantlets exposed to 50 mM NaCl decreased by 16.31 % over that of control. Chl_b, TC and C_{x+c} contents declined significantly by 24.38 %, 24.84 % and 27.78 %, respectively, over the control when plantlets were subjected to 100 mM NaCl for 28 days (Table 2). A positive correlation was observed between TC and P_n (Fig. 3B). The P_n in salt stressed plantlets declined significantly in relation to salt concentration in the culture medium (Fig. 2C). On the other hand, a positive relationship was observed between P_n and plant dry weight (Fig 3C). Overall, photosynthetic pigments in salt stressed plantlets of cactus were significantly damaged leading to reduction in P_n and resulting in growth inhibition.

Photosynthetic pigments, i.e. Chl_a, Chl_b, TC and C_{x+c}, are highly sensitive to salt stress and dropped significantly in salt-stresses plantlets. Likewise, chlorosis or pigment degradation was clearly evident when Jerusalem artichoke cv. 'NY1' was grown under 150 mM NaCl for 20 days (Huang et al. 2012). In contrast, chlorophyll content in salt stressed cactus pear was enhanced under NaCl (Silva-Ortega et al. 2008). Net CO_2 uptake or net photosynthetic rate (P_n) declined significantly when cactus

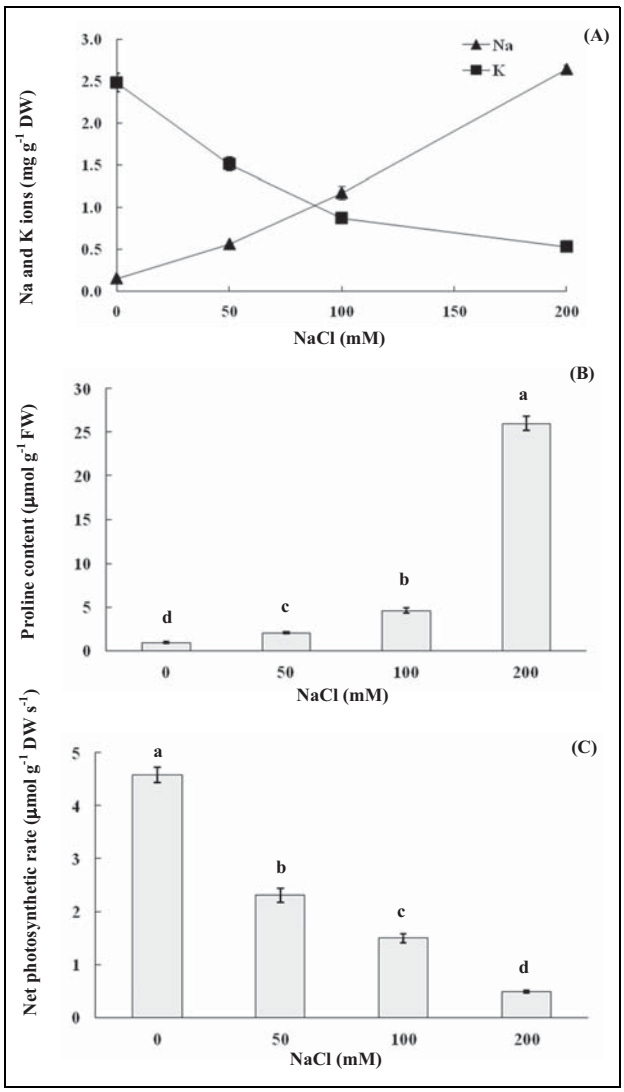


Fig. 2. Sodium (Na^+) and potassium (K^+) (A), proline (B), and net photosynthetic rate (C) of *Echinopsis* spp. grown *in vitro* under 0, 50, 100 and 200 mM NaCl for 28 days. Error bars represent ± SE. Different letters in each bar show significant difference at $P \leq 0.01$ by the Tukey's HSD.

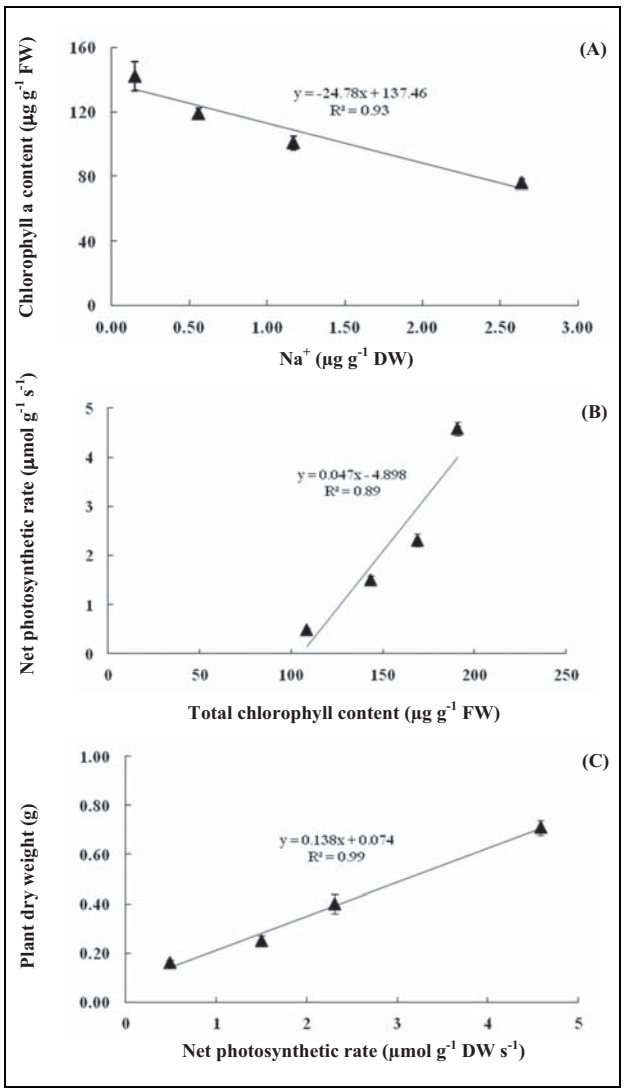


Fig. 3. Relationship between Na^+ and chlorophyll a content (A), total chlorophyll content and net photosynthetic rate (B) and net photosynthetic rate and plant dry weight (C) of *Echinopsis* spp. grown *in vitro* under 0, 50, 100 and 200 mM NaCl for 28 days. Error bars represent ± SE.

Table 2. Chlorophyll a (Chl_a), chlorophyll b (Chl_b), total chlorophyll (TC) and total carotenoids (C_{x+c}) contents in *Echinopsis* cactus grown *in vitro* under 0, 50, 100 and 200 mM NaCl for 28 days. Data presented as mean ± SE.

NaCl (mM)	Chl_a (µg g^{-1} FW)	Chl_b (µg g^{-1} FW)	TC (µg g^{-1} FW)	C_{x+c} (µg g^{-1} FW)
0	142.2 ± 9.2 a	56.6 ± 2.2 a	190.8 ± 11.2 a	3.6 ± 0.1 a
50	119.0 ± 3.1 b	49.6 ± 1.1 ab	168.7 ± 4.2 ab	2.9 ± 0.1 ab
100	100.6 ± 4.4 c	42.8 ± 1.6 b	143.4 ± 5.8 bc	2.6 ± 0.1 bc
200	76.0 ± 2.5 c	32.5 ± 0.3 c	108.5 ± 2.7 c	1.9 ± 0.2 c

Different letters in each column show significant difference at $P \leq 0.01$ by the Tukey's HSD.

plants were subjected to salt stress. In CAM plants, the CO_2 assimilation occurs in the dark; so, the net CO_2 uptake by cactus is assayed in the night. In cactus pear, P_n reduction was observed in plants subjected to 50–200 mol m^{-3} NaCl (Nerd et al. 1991). Likewise, the P_n in salt stressed plants of Jeruslalem artichoke cvs. 'NY1'and 'NY2' declined when plants were exposed to 30–150 mM NaCl (Huang et al. 2012).

In conclusion, exogenous NaCl in the culture medium induces accumulation of Na^+ in the salt stressed cactus plantlets resulting in photosynthetic pigment degradation, net photosynthetic rate reduction and overall growth reduction. Free proline enrichment was one of candidate osmolytes that helps to protect the plant cells exposed to low water potential derived from salt stress. Root initiation in cactus plantlets was sensitive to salt toxicity, and may act as good indicator of salt stress in cactus species.

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