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**ORIGINAL ARTICLE**

# Influence of NaCl on Biochemical Parameters of Two Cultivars of *Stevia rebaudiana* Regenerated *in vitro*

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Soil salinity occupies a prominent place among the soil problems that threaten the sustainability of agriculture over a vast area in the world. It affects plant morpho-physiology and ultimately leads to reduction in productivity. It is essential to test important medicinal plants for their salinity tolerance as research efforts aim to explore economic benefits under saline conditions. Keeping in view the importance of *Stevia* and salinity, present study had been designed to investigate the effect of salinity on biochemical parameters in two *Stevia* genotypes. Two node microcuttings were subjected to MS media supplemented with different NaCl concentrations (0, 25, 50, 75, 100, 125 mM). Chlorophyll amount was observed to be decreased as compared to sugars, proline and phenols with increased salt concentrations.

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*Stevia*, a member of family Asteraceae, is native to Paraguay, Brazil, Venezuela and Colombia. It is an important non caloric sweetener medicinal plant, used for pharmaceutical, food and cosmetic industries. Now it is being cultivated in Japan, Taiwan, Philippines, Hawaii, Malaysia and overall South America for food and pharmaceutical products (Ahmed *et al.,* 2007). It is commonly known as *Stevia*, honey leaf, ya wan, sweet grass etc. It is one of the most promising herb used in the treatment of hypoglycaemia, indigestion, skin toning, healing heart disease. It is used as a table top sweetener, in soft drinks, baked goods, pickles, fruit juices, tobacco products, confectionery goods, jams and jellies, candies, yogurts, pastries, chewing gum and sherbets (Ali *et al.,* 2010).

Soil salinity is one the most important stress factor which limits plant growth and productivity (Jamil *et al.,* 2012). Salinity mainly occurs in arid and semi-arid conditions (Ehert and Ho, 1986) when the precipitation is not enough to leach the excess of soluble salts from the root zone and poor quality of water is used for irrigation (Mohammad *et al.,* 1998). Exposure of plants to a stressful

environment during various developmental stages appears to induce various physiological and developmental changes (Islam *et al.,* 2008). Plants cultured under salt stress show high chlorophyll degradation and high proline accumulation. The effect of salt stress on biochemical parameters on *in vitro* regenerated plants of *Stevia* has not yet been investigated. The objective of this investigation was to elucidate the effect of NaCl on biochemical parameters of *in vitro* regenerated plantlets of *Stevia* genotypes.

## MATERIALS AND METHODS

### Total soluble sugars

The amount of total soluble sugars was estimated using Anthrone reagents as given by Thimmaiah (2004). One hundred mg sample was taken in a boiling tube and hydrolyzed with 5 ml 2.5 N HCl in a water bath for 3 h. It was then neutralized with solid sodium carbonate until effervescence ceased. The volume was made to 100 ml followed by centrifuge at 5000 rpm for 10 min. The supernatant was collected and 1 ml sample was taken for analysis. Four ml Anthrone reagent was added to aliquot and heated for one min. in water bath (70°C). The sample was then rapidly cooled and the change of green to dark green colour was read at 630 nm against blank.

### Reducing sugars

Reducing sugars were extracted from leaf samples of both the cultivars viz. CIM madhu and CIM mithi and Somogyi (1951) method was used. The reducing sugars were determined as mg/g DW.

### Proline content

The leaf tissue proline content was measured following the method described by Bates *et al.* (1973). A homogenized fresh leaf tissue (0.5 g) was added in 10 ml of 3% sulfo-salicylic acid. Homogenates of fresh leaf samples were filtered through Whatman No. 2 filter paper. Two ml of the filterate was taken in a test tube containing 2 ml of acid ninhydrin solution (1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml of 6 M orthophosphoric acid). Then, 2 ml of glacial acetic acid was added in a test tube containing filtrate and heated for 1 h at 100°C. Test tubes were then shifted in an ice bath to terminate the reaction. Reaction mixture was then extracted with 10 ml toluene and mixed vigorously by passing a continuous air stream for 1-2 minutes. The absorbance was noted at 520 nm.

### Total Phenols

The leaf tissue proline content was measured following the method described by Malik and Singh (1980). Thus, 100 mg of fresh leaf material was homogenized by adding by adding 80 per cent ethanol and then heated in a water bath at 58°C for 60 min. The absorbance was measured at 650 nm in a spectrophotometer.

### Chlorophyll content

The fractions of pigments (chlorophyll a, chlorophyll b and Total chlorophyll) were estimated using the spectrophotometric method recommended by Arnon (1949). A pinch of $CaCO_3$ was added to avoid the destruction of chlorophyll and other pigments. Extraction has to be carried out under dim light to avoid photo oxidation of the pigments. It was centrifuged in a Rim centrifuge at low speed (5000 rpm) for about 20 min. Chlorophyll contents concentrations were calculated as mg/g FW at 663 and 645 nm.

### Statistical analysis

Data were analyzed for significance using one-way analysis of variance (ANOVA) and the differences contrasted using a Duncan's multiple range test (DMRT) at $p \leq 0.05$. All statistical

analyses were performed using the Statistical Package for Social Sciences (SPSS, version 11.5).

## RESULTS

### Soluble sugars

Addition of NaCl to proliferation medium caused an increase in total soluble sugars on *in vitro* raised shoots of both the cultivars (Figure 1). The lowest content (55.07 mg/g) of total soluble sugars was found in regenerants cultured on MS medium free from NaCl. The highest amount of soluble sugars (93.54 mg/g) was found in cv. CIM madhu at 75 mM NaCl. It was determined that total soluble sugars in both the cultivars increased linearly.

### Reducing sugars

Changes in reducing sugars in different cultivars are more or less similar to changes in total sugars. The highest level of reducing sugars (55.78 mg/g) was recorded in media containing 75 mM NaCl whereas, minimum amount of reducing sugars (22.86 mg/g) was found on media free from NaCl. Among the two *Stevia* cultivars, cv. CIM madhu produced higher reducing sugars (Figure 2).

### Proline

It is generally assumed that proline is acting as a compatible solute in osmotic adjustment. The highest value of (22.26 µg/g) proline was observed at the concentration of 75 mM of NaCl. According to results presented in Figure 3, proline content of both the cultivars increase significantly ($p<0.05$) with the increase of salt concentration on culture media (MS). Minimum concentration of proline was found on medium free of NaCl.

### Total phenols

As the level of NaCl was increased in the medium, the total phenols were also increased concurrently (Figure 4). The highest level of phenols was recorded in cv. CIM madhu (22.42 mg/g) cultured on medium supplemented with 75 mM NaCl. Minimum concentration (16.94 mg/g) of total phenols was found on media without NaCl.

### Chlorophyll contents

The Chlorophyll contents were found to be decreased in leaves produced under stress conditions (Table 1). Chlorophyll a content decreased with the increase in concentration of NaCl. The highest amount of chlorophyll b (1.23 mg/g) was found in cv. CIM madhu when cultured on medium free from NaCl. Minimum chlorophyll b (0.05 mg/g) was observed at 75 mM NaCl. The highest amount of total chlorophyll (3.27 mg/g) was recorded in cv. CIM mithi cultured on medium without NaCl.

**Table 1.** Effect of different sodium chloride concentrations on chlorophyll contents (mg/g FW) of *S. rebaudiana* cultivars.

| Treatment | CIM madhu | | | CIM mithi | | |
|---|---|---|---|---|---|---|
| | Chlorophyll a | Chlorophyll b | Total chlorophyll | Chlorophyll a | Chlorophyll b | Total chlorophyll |
| Control | 2.02±0.018$^{a}$ | 1.23±0.023$^{a}$ | 3.01±0.021$^{a}$ | 2.05±0.006$^{a}$ | 0.89±0.047$^{a}$ | 3.27±0.060$^{a}$ |
| 25 mM | 1.64±0.184$^{b}$ | 0.96±0.018$^{b}$ | 2.62±0.021$^{b}$ | 1.62±0.059$^{b}$ | 0.87±0.054$^{a}$ | 2.49±0.105$^{b}$ |
| 50mM | 1.40±0.049$^{c}$ | 0.96±0.032$^{b}$ | 2.36±0.020$^{c}$ | 1.30±0.058$^{c}$ | 1.01±0.007$^{a}$ | 2.31±0.010$^{b}$ |
| 75mM | 0.24±0.053$^{d}$ | 0.05±0.037$^{c}$ | 0.30±0.020$^{d}$ | 0.23±0.025$^{d}$ | 0.04±0.009$^{b}$ | 0.28±0.032$^{c}$ |
| 100mM | - | - | - | - | - | - |
| 125mM | - | - | - | - | - | - |
| **LSD (p<0.05)** | 0.018 | 0.001 | 0.039 | 0.003 | 0.002 | 0.007 |

Values represent mean ± standard error.
Means values within a column sharing the same subscript are not significantly different at $P<0.05$ according to Duncan's Multiple Range Test.

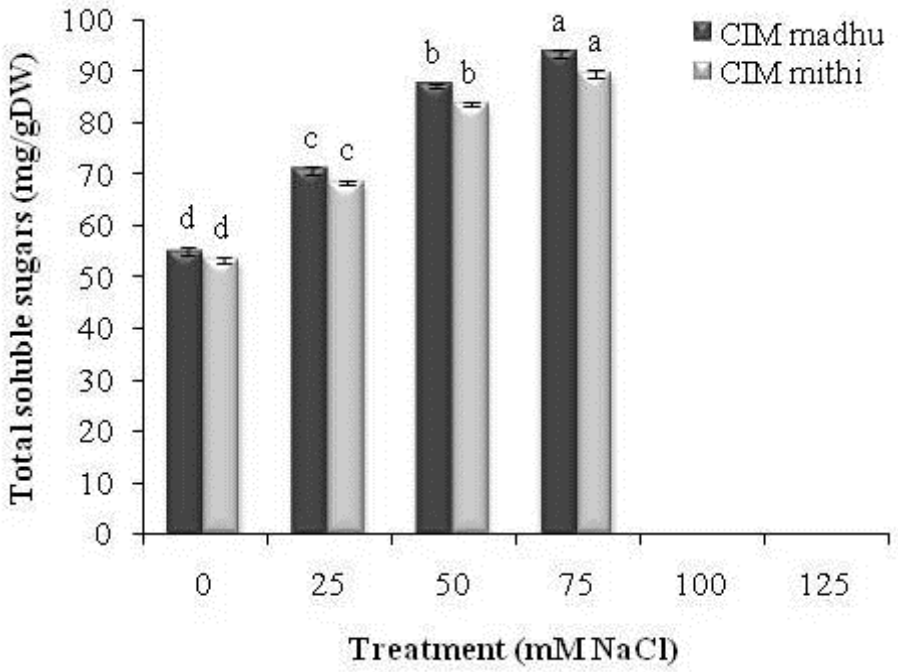


**Figure 1.** Total soluble sugars of two S*. rebaudiana* cultivars cultured on MS medium fortified with NaCl (00, 25, 50, 75,100 and 125mM). Bars carrying different letters are significantly different at P< 0.05.

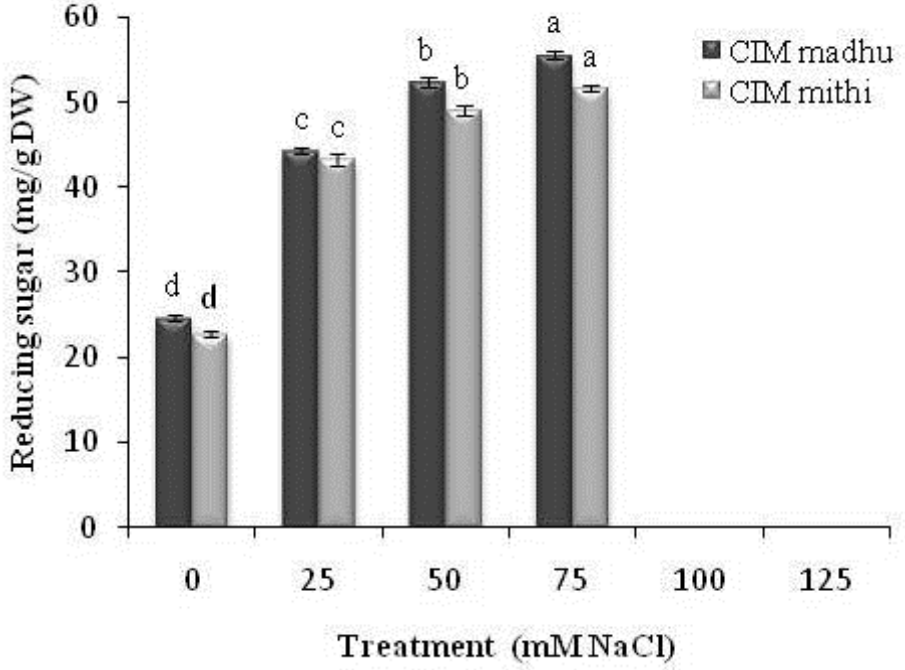


**Figure 2.** Reducing sugars of two S*. rebaudiana* cultivars cultured on MS medium fortified with NaCl (00, 25, 50, 75,100 and 125mM). Bars carrying different letters are significantly different at P< 0.05.

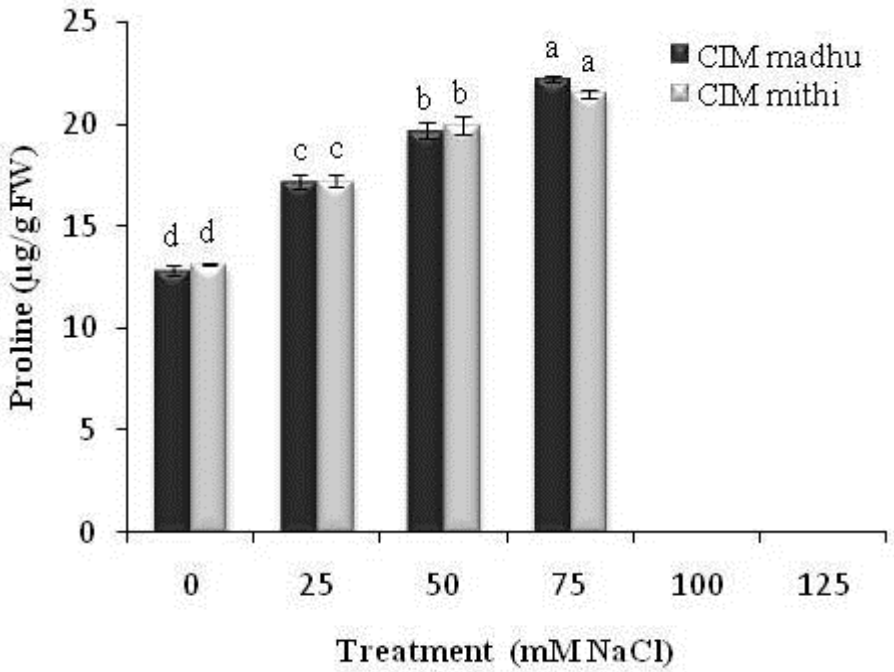


**Figure 3.** Proline of two S*. rebaudiana* cultivars cultured on MS medium fortified with NaCl (00, 25, 50, 75,100 and 125mM). Bars carrying different letters are significantly different at P< 0.05.

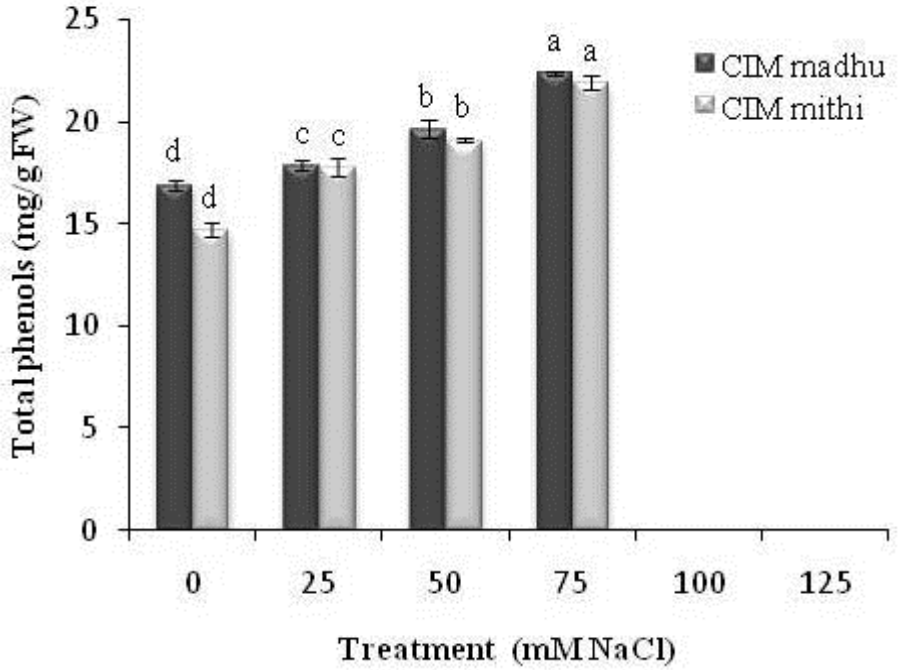


**Figure 4.** Total phenols of two S*. rebaudiana* cultivars cultured on MS medium fortified with NaCl (00, 25, 50, 75,100 and 125mM). Bars carrying different letters are significantly different at P< 0.05.

## DISCUSSION

Biochemical studies were under taken when the regenerated shoots were transferred to the NaCl treated medium. The addition of sodium chloride to the culture medium resulted in marked alteration of biochemical constituents and accordingly their levels varied with NaCl concentrations.

Sodium chloride treatment induced stimulatory effect on the accumulation of soluble sugars. The accumulation of soluble carbohydrates in plants has

been widely reported as a response to salinity or drought (Popp and Smirnoff, 1995; Murakeozy *et al.,* 2003). Maximum amount of total soluble sugars was found at 100 mM NaCl. Significant increases in soluble sugars content of shoots of wheat cultivars were reported by Zheng *et al.* (2008) in response to NaCl stress. The accumulation of sucrose under salt stress supported the well-established role of the sugars as an osmoprotectant that stabilizes cellular membranes and maintains turgor (Whittaker *et al.,* 2001; Jouve *et al.,* 2004). In the present studies, the highest quantity of proline was observed at 75 mM NaCl in both the cultivars. Many authors have reported the increase in proline accumulation under salt stress in different plants such as pigeon pea (Waheed *et al.,* 2006), *Sesamum indicum* (Koca *et al.,* 2007), barley (Sadeghi, 2009), maize (Chaum and Kirdmanee, 2009), jojoba plant (Fayek *et al.,* 2010)**.** Total phenols were also found to increase with increasing concentration of NaCl. It was found to be maximum in the shoots implanted on proliferation media containing 75 mM NaCl in both the cultivars. Total phenols play a significant role in the regulation of plant metabolic processes and overall plant growth (Lewis and Yamamoto, 1990). It has been shown in some studies that polyphenols synthesis depends on abiotic factors (Ksouri *et al.,* 2008; Megdiche *et al.,* 2009; Naffeti *et al.,* 2011). Salinity induced disturbances of the metabolic process leading to an increase in phenolic compounds have been reported by Ayaz *et al.* (2000) and Radi *et al.* (2013).

In the present studies, the chlorophyll contents decreased as the NaCl concentration increased in both the cultivars. Change in chlorophyll contents due to salinity is the most obvious biochemical response (Erturk *et al.,* 2007; Sherif, 2012). Chlorophyll contents in salt stressed plants were significantly decreased depending on NaCl concentration (Jamil *et al.,* 2012). This trend has previously been reported by (Singh *et al.,* 2000; Khawale *et al.,* 2003). The depressive effect of salt stress on chlorophyll biosynthesis may be due to the formation of proteolytic enzymes such as chlorophyllase which is responsible for the chlorophyll degradation (Sabater and Rodriguez, 1978) and damaging the photosynthetic apparatus (Singh and Jain, 1981; Yasseen, 1983).

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