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Salt stress induced effects on biochemical parameters in etiolated maize leaf segments during greening

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Abstract

Treatment of etiolated maize leaf segments with 0-300 mM NaCl during greening decreased the osmotic potential of leaf sap significantly. Na^+ content of the leaf tissue increased significantly on supplying different concentrations of salt. Relative water content of the leaf tissue was marginally affected by the supply of NaCl. Total protein and RNA content of the maize leaf segments gradually increased when treated with lower concentrations of NaCl and subsequently decreased at higher concentrations. Decrease in DNA content was noted with increasing salt treatment in a concentration dependent manner. SDS-PAGE analysis of salt treated maize leaf tissue revealed appearance of one protein band (approx. 73 kD) in samples incubated with ≥50 mM NaCl compared to control. Protein identification performed with the Mascot search engine in NCBI database indicated extensive homology of this protein with chloroplast heat shock protein 70 of *Cenchrus americanus* (Protein score: 295), hypothetical proteins SELMODRAFT_267815 of *Selaginella moellendorffii* (Protein score: 125), VITISV_000728 of *Vitis vinifera* (Protein score: 118) and SELMODRAFT_230659 of *Selaginella moellendorffii* (Protein score: 51). The results revealed that the dark grown maize leaf segments exhibit high degree of stress due to NaCl treatment, which affects the biochemical parameters governing the metabolic activities of the leaf tissue. Further, the stress induced proteins are being synthesized in leaf tissue in response to salt stress.

Keywords: *Salt stress, Maize leaf, Protein, NaCl*

1. Introduction

Soil salinity is a major abiotic factor limiting plant growth and development at all growth stages (Khoshsokan *et al.*, 2012). It affects the plants in two ways: higher concentrations of salts in the soil make it harder for roots to extract water, i.e., osmotic stress, and secondly high salt level within the plant may be harmful, i.e., specific ion toxicity. Changes in water relations have significant influence as salinity can be seen in many plants which undergo osmotic regulation on exposure to salt stress by increasing the negativity of the osmotic potential of leaf sap (Kaymakanova and Stoeva, 2008; Kaymakanova *et al.*, 2008; and Gama *et al.*, 2009). Further, less absorbed water means less water content, indicated by Relative Water Content (RWC) and a high degree of sclerophylly. In this respect, decreased leaf succulence of maize (Kholova *et al.*, 2009) and mungbean

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(Kabir *et al*., 2004) have been reported under saline environment. During long-term exposure to salinity, plants experience ionic stress, which results in premature senescence of leaves and toxicity symptoms, such as, chlorosis, necrosis, etc. (Munns, 2002). Ion homeostasis is generally ascribed to high K^+/Na^+ ratio in the cytoplasm (Flowers, 2004). Under saline conditions, external Na^+ negatively impacts intracellular K^+ influx, so that plants may acquire Na^+ at the cost of Ca^{2+} and K^+ (Munns and Tester, 2008). High Na^+ accumulation in plant tissues leads to membrane damage as well as interference with metabolic activities, such as, protein synthesis and enzyme activity in the cytosol (Katerji *et al*., 2004; and Arzani, 2008).

All major metabolic processes, such as, protein synthesis, production of nucleic acids and lipids as well as energy metabolism is affected by salt stress (Kapoor and Srivastava, 2010; and Sobhanian *et al*., 2010). Qualitative as well as quantitative changes in proteins of plants have been reported under the saline condition (Ashraf and Harris, 2004). Amirjani (2010) has reported an intensive decrease in total protein content at higher salt concentration in rice seedlings. Salt stress induced DNA degradation in meristematic cells of barley roots has been observed (Katsuhara and Kawasaki, 1996). *Zea mays* is one of the World's most important crop plant due to its agronomic importance. It is being used as food and fuel for human being and feeds for livestock and poultry (Wattoo *et al*., 2009). The present study aimed to analyze the biochemical changes in maize leaf tissue subjected to salt stress by using NaCl.

2. Materials and Methods

2.1. Plant Material, Growth Conditions and Treatment

Seeds of *Zea mays* L., cv. Ganga safed-2 were surface sterilized with 0.1% $HgCl_2$ for 1-2 min and then washed thoroughly with distilled water. Seedlings were raised in small plastic pots containing acid washed sand in continuous darkness for 6-7 days at 25 ± 3 °C. They were watered on alternate days with half strength Hoagland's solution without Nitrogen.

For various experiments, primary leaves from uniformly grown maize seedlings were cut into about 0.5 x 0.5 cm^2 pieces and treated with different concentrations of NaCl (50, 100, 150, 200 and 300 mM) for 24 h in continuous intensity of light (40 Wm^{-2}) at 25 ± 3 °C. Treated leaf segments were thoroughly washed with distilled water prior to analysis.

2.2. Biochemical Analyses

Osmotic potential of cell sap was measured according to Kucukkomurcu (2011), using Osmometer (Wescor Vapro 5520, USA).

Na^+ and K^+ content were estimated by flame photometer (ESICO model 1385) according to the method of Watson and Isaac (1990).

The RWC was measured according to the method of Barr and Weatherly (1962), using the following equation:

$$RWC(\%) = \frac{(FW - DW)}{(TW - DW)} \times 100$$

where, *FW* = Fresh weight, *DW* = Dry weight, and *TW* = Turgid weight.

The protein content was estimated using Folin and Ciocalteu's phenol reagent by the method of Lowry *et al*. (1951).

The SDS-PAGE analysis of the extracted protein was performed according to the procedure of Laemmli (1970). Mass spectrometric analysis of the selected protein bands from dark grown maize leaf segments treated with 200 mM and 300 mM NaCl was carried out by Peptide Mass Fingerprinting (PMF) and MS/MS analysis on 4800 plus MALDI TOF/TOF platform (Applied Bios Stems®, USA). The digestion of selected protein band and extraction of peptides was carried out as described by Upadhyay *et al*. (2010).

2.2.1. Data Acquisition and Analysis

Monoisotopic peptides obtained from MALDI TOF/TOF were analyzed by the 4000 Series Explorer Software (version 3.5, ABI, USA). Based on the mass signals, protein identification was performed with the Mascot search engine (http://www.matrixscience.com) in the NCBI nr database.

The total RNA content was estimated using orcinol reagent by the method of Webb and Levy (1958).

The total DNA content was estimated using diphenylamine (DPA) reagent by the method of Gendimenico *et al*. (1988).

3. Statistical Analysis

Data presented in the study are average of at least four independent experiments with ± S.E. Significance of difference obtained for various treatments was tested by Student's '*t*' test.

4. Results and Discussion

Supply of 0-300 mM NaCl to the etiolated maize leaf segments during greening decreased the osmotic potential of leaf sap significantly with increasing concentration (Table 1). Correlation analysis was performed using Microsoft Excel chart type X-Y scatter between NaCl concentrations and osmotic potential, which yielded a highly significant R^2-value of 0.98 (Table 1).

Table 1: Effect of salt stress imposed by using NaCl on osmotic potential in etiolated maize leaf segments during greening

Leaf segments from dark grown maize seedlings were treated with varying concentrations of NaCl in continuous light for 24 h at 25 ± 3 °C

NaCl conc. (mM)	Osmotic potential (MPa)
0	–0.98 ± 0.03
50	–1.20 ± 0.04**
100	–1.36 ± 0.02**
150	–1.56 ± 0.02**
200	–1.68 ± 0.02***
300	–1.89 ± 0.06***
R^2-value	0.98

Note: Level of significance: '*p*' values <0.01** and <0.001*** compared with control.

When excised etiolated maize leaf segments were treated with varying concentrations of NaCl during greening, the Na^+ content of the leaf tissue increased substantially and significantly (Table 2). There was about 17-fold increase in Na^+ content of leaf segments on treatment with 300 mM NaCl. The potassium content of leaf tissue increased gradually from 50-150 mM concentration of NaCl; thereafter it is decreased (Table 2). As a

Table 2: Effect of salt stress imposed by using NaCl on Na^+ content, K^+ content and Na^+/K^+ ratio in etiolated maize leaf segments during greening

Leaf segments from dark grown maize seedlings were treated with varying concentrations of NaCl in continuous light for 24 h at 25 ± 3 °C

NaCl conc. (mM)	Sodium content (μg g^{-1} FW)	Potassium content (μg g^{-1} FW)	Na^+/K^+ ratio
0	213 ± 13 (100)	463 ± 25 (100)	0.46 (100)
50	674 ± 21***(316)	486 ± 30 (108)	1.39***(302)
100	1038 ± 32***(487)	548 ± 35**(118)	1.89***(411)
150	1504 ± 52***(705)	576 ± 32**(124)	2.61***(567)
200	2514 ± 56***(1179)	324 ± 15**(70)	7.76***(1687)
300	3522 ± 59***(1654)	266 ± 6***(57)	13.26***(2883)
R^2-value	0.98	0.45	0.89

Note: Values relative to control are given in parentheses; and Level of significance: '*p*' values <0.01**, and <0.001*** compared with control.

Table 3: Effect of salt stress imposed by using NaCl on fresh weight, turgid weight, dry weight and relative water content in etiolated maize leaf segments during greening

Leaf segments from dark grown maize seedlings were treated with varying concentrations of NaCl in continuous light for 24 h at 25 ± 3 °C

NaCl conc. (mM)	Fresh weight (mg)	Turgid weight (mg)	Dry weight (mg)	RWC (%)
0	216 ± 4 (100)	224 ± 4 (100)	19.4 ± 0.3 (100)	95.9 ± 1.6 (100)
50	221 ± 4 (105)	233 ± 4 (104)	20.7 ± 0.7 (107)	94.5 ± 1.3 (101)
100	219 ± 2 (104)	238 ± 2 (106)	19.9 ± 0.5 (103)	91.0 ± 1.1 (97)
150	212 ± 2 (100)	239 ± 2 (106)	18.8 ± 0.3 (97)	88.0 ± 1.0 (94)
200	211 ± 2 (100)	233 ± 2 (104)	18.2 ± 0.5 (94)	89.6 ± 1.1 (96)
300	200 ± 2 (95)	191 ± 4* (86)	13.9 ± 0.6* (72)	105 ± 2.7 (110)
R^2-value	0.78	0.36	0.76	0.83

Note: Values relative to control are given in parentheses; and Level of significance: '*p*' values <0.05*, compared with control.

result of these changes in ion content, the Na^+/K^+ ratio increased gradually initially, whereas substantially at higher concentrations. A highly significant R^2-value of 0.98 was obtained from correlation analysis with Na^+ content and 0.89 for Na^+/K^+ ratio (Table 2).

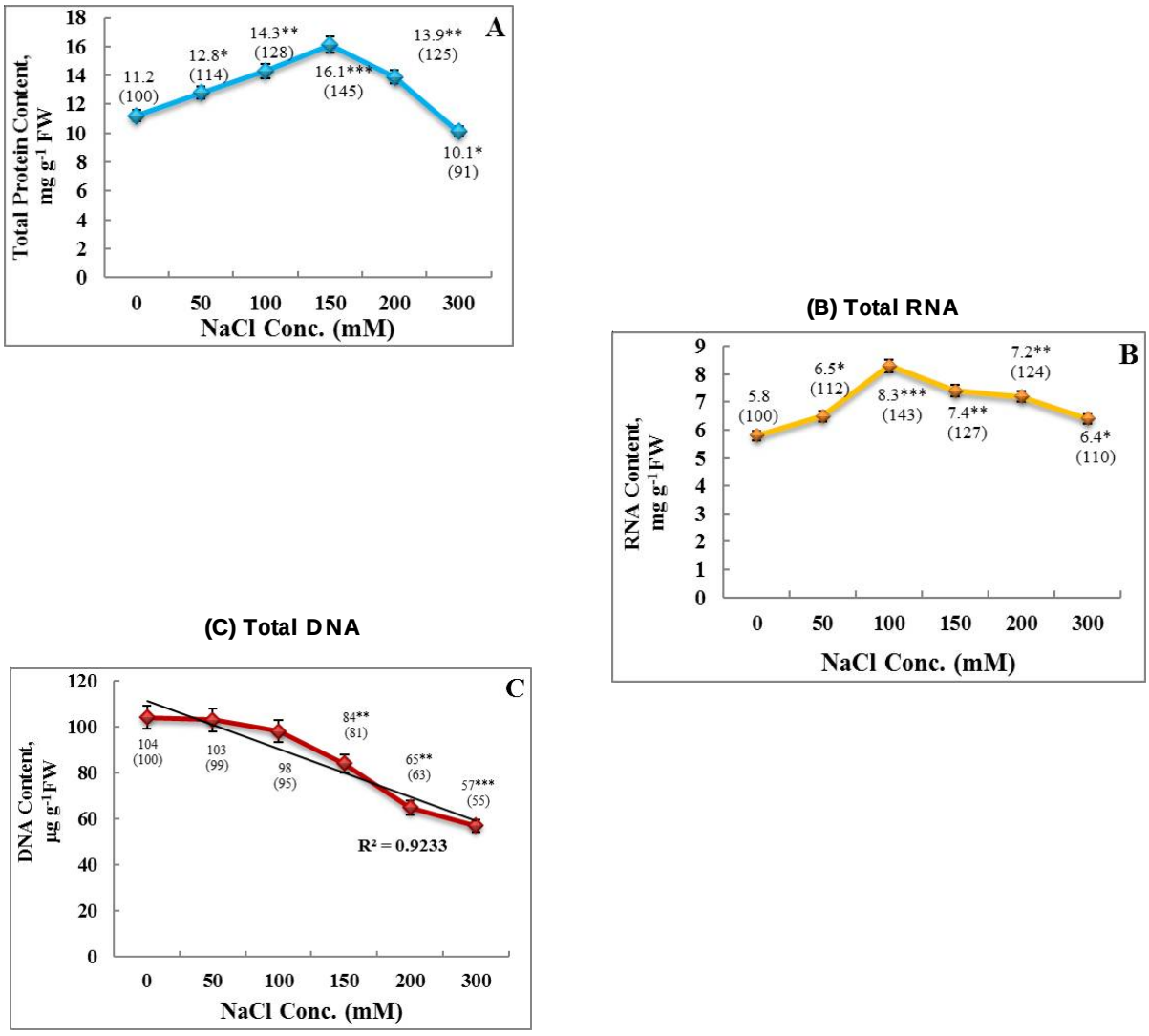
Treatment of etiolated maize leaf segments with 300 mM NaCl during greening decreased the turgid weight and dry weight considerably, while, almost no change in fresh weight was noted. Further, at 300 mM NaCl, the turgid weight was found to be lower than the fresh weight (Table 3). RWC remained almost unchanged at all the concentrations of NaCl (Table 3).

The total protein content of the maize leaf segments gradually increased with the supply of 50-150 mM NaCl and thereafter decreased (Figure 1A). There was an increase in RNA content of the leaf tissue also up to 100 mM NaCl, and then a decrease from 150-300 mM salt concentration. However, at the highest concentration of NaCl, RNA content was maintained higher than the control (Figure 1B). Concentration dependent decrease in DNA content was noted with increasing salt treatment (Figure 1C).

Protein profile obtained with 12% SDS-PAGE for leaf segments from dark grown maize seedlings treated with NaCl is shown in Figure 2. The SDS-PAGE analysis of salt treated maize leaf tissue revealed the appearance of one distinct protein band (approx. 73 kDa) in samples incubated with ≥50 mM NaCl as compared to control (Figure 2).

The excised protein band was subjected to MS-MS analysis and data were analyzed by mascot search against the reported protein database of NCBI. Upon search, extensive homology (Protein score: 295, Figure 3) was found with **gi|145388994** chloroplast heat shock protein 70 of *Cenchrus americanus* (Nominal Mass: 73137; Number of mass values matched: 6(2); sequences 6(2)), **gi|302786294** hypothetical protein SELMODRAFT_267815 of *Selaginella moellendorffii* (Protein score: 125; Nominal Mass: 70734; Number of mass values matched: 4(1); **gi|302786294** sequences 4(1)), hypothetical protein VITISV_000728 of *Vitis vinifera* (Protein score: 118; Nominal Mass: 74472; Number of mass values matched: 4(0); sequences 4(0)) and **gi|302764140** hypothetical protein SELMODRAFT_230659 of *Selaginella moellendorffii* (Protein score: 51; Number of mass values matched: 1(1); sequences 1(1)). Proteins matching the same set of peptides also found with **gi|242038471** hypothetical protein SORBIDRAFT_01g011310 of *Sorghum bicolor*, **gi|413933381** hypothetical protein ZEAMMB73_095591, **gi|414872232** hypothetical protein ZEAMMB73_352617 and **gi|226495067** uncharacterized protein LOC100280354 from *Zea mays* (Protein score: 295). Sequences of peptides matched with different mass values are presented in Tables 4A, 4B, 4C and 4D.

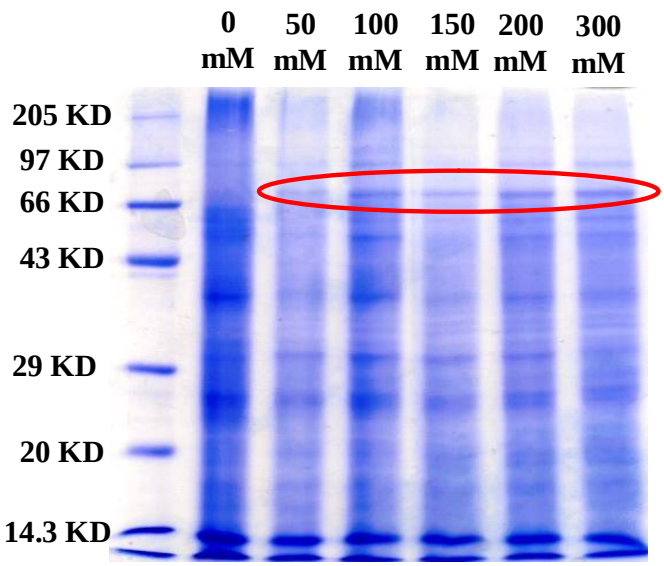
Plants maintain the pressure potential by increasing the negativity of osmotic potential of the leaf sap when they are exposed to salt stress (Gama *et al.*, 2009). The results of the present study are in agreement with this statement (Table 1). Thus, by decreasing the osmotic potential of leaf sap below the osmotic potential of the



Note: Leaf segments from dark grown maize seedlings were treated with varying concentrations of NaCl in continuous light for 24 h at 25 ± 3 °C; Values relative to control are given in parentheses; Level of significance: '*p*' values <0.05*, <0.01**, and <0.001*** compared with control.

Figure 1: Effect of salt stress imposed by using NaCl on total protein, total RNA and DNA content in etiolated maize leaf segments during greening

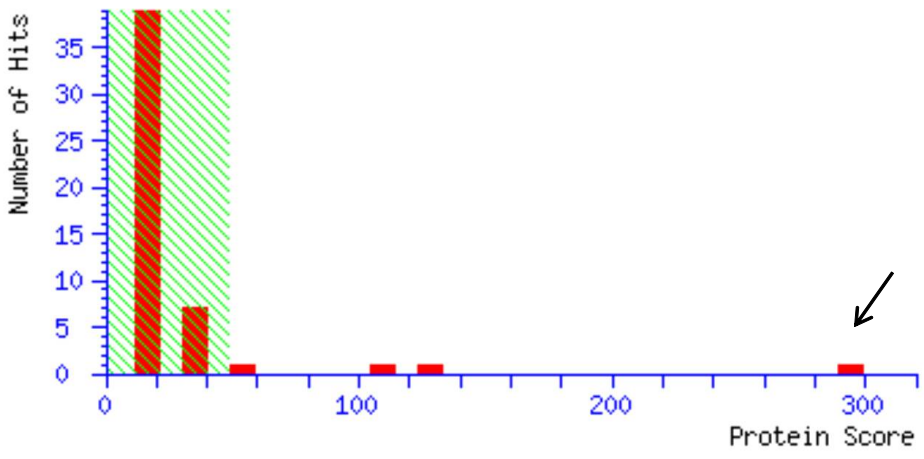
surrounding solution, the leaf tissue maintains its tolerance towards the harmful effect of salt accumulation during salt stress, and thus, increases its ability to absorb water. The decrease in osmotic potential of leaf sap with salinity has been reported in bean plants (Stoeva and Kaymakanova, 2008; and Amira and Qados, 2011). Both K^+ and Na^+ ions are essential for osmotic adjustment; however, their lower and higher levels can have adverse effects on plants. In the present study, supply of 0-300 mM NaCl to excised maize leaf segments during greening increased the Na^+ content with increasing concentration (Table 2). The potassium content of the leaf tissue increased at lower concentrations of NaCl and then decreased at higher concentrations (Table 2). Such changes in ion content imply that higher levels of salinity and Na^+ have a negative effect on absorption and accumulation of K^+. It has been suggested that the plant tolerance is characterized by distinctly lower Na^+/K^+ ratio. It can be used to predict tolerance or sensitivity in wheat varieties (Sairam *et al.*, 2002). Hence, it is possible that up to 150 mM concentration of NaCl, tolerance is observed towards salt stress and subsequently it is lost (Table 2). Increase in Na^+ content, decrease in K^+ content and highly increased Na^+/K^+ ratio has been reported in both shoot and root of rice seedlings under the condition of salt stress (Turan and Tripathy, 2015). Further, increased Na^+/K^+ ratio can cause a severe ionic imbalance which may lead to oxidative stress (Turan and Tripathy, 2015).



Note: Leaf segments from dark grown maize seedlings were treated with varying concentrations of NaCl in continuous light for 24 h at 25 ± 3 °C.

Figure 2: Effect of salt stress imposed by using NaCl on protein profile in excised etiolated maize leaf segments during greening

Maintenance of water balance is a fundamental phenomenon for normal growth of plant under stressful environment. Disturbances in water balance leads to impaired functions hence reduced growth. In this study, decrease in dry weight is noted with the supply of 300 mM NaCl to the etiolated maize leaf segments during greening, however, no change in fresh weight was found (Table 3). Marked reduction in fresh and dry masses has been shown in two maize cultivars under salt stress conditions (Cicek and Cakirlar, 2002; and Hussain *et al.*, 2013). Further, the decrease in seedling dry weight has also been demonstrated by NaCl in rice (Turan and Tripathy, 2015). RWC parameter can be used to select high yielding genotypes that maintain cell turgor under water stress environment to give relative high yield (Bayoumi *et al.*, 2008). In the present study, almost no change in RWC is observed when maize leaf segments were treated with NaCl (Table 3).



Note: It shows results from a protein database search with the MS spectrum peak list data using Mascot. The top score (295) matches the correct *Cenchrus americanus* accession.

Figure 3: MALDI-TOF mass spectrum histogram

To analyze the effect of salt stress induced by NaCl on overall metabolic activities, total RNA and total protein content were measured in leaf segments. A marginal reduction in protein content was observed with the supply of 300 mM NaCl; however, the higher level was found at lower concentrations of salt (Figure 1A). Thus, higher protein content due to NaCl supplementation may be due to the synthesis of stress induced proteins. The SDS-PAGE analysis of salt treated maize leaf tissue revealed one protein band (approx. 73 k

Table 4A: The MALDI TOF mass spectrum sequence of peptides matched with gi|145388994 chloroplast heat shock protein 70 of *Cenchrus americanus* (Protein score: 295)

S. No.	Peptides identified by MS/MS	Mr (expt.)	Mr (calc.)
1.	K.DIDEVILVGGSTR.I	1372.9911	1372.7198
2.	K.QFAAEEISAQVLR.K	1461.0489	1460.7623
3.	K.AVITVPAYFNDSQR.T	1580.1024	1579.7995
4.	R.QAVVNPENTFFSVKR.F	1735.2343	1734.9053
5.	K.LQFKDIDEVILVGGSTR.I	1889.3760	1889.0258
6.	K.SEVFSTAADGQTSVEINVLQGER.E	2436.6431	2436.1769

Table 4B: The MALDI TOF mass spectrum sequence of peptides matched with gi|302786294 hypothetical protein SELMODRAFT_267815 of *Selaginella moellendorffii* (Protein score: 125)

S. No.	Peptides identified by MS/MS	Mr (expt.)	Mr (calc.)
1.	K.QFAAEEISAQVLR.K	1461.0489	1460.7623
2.	K.AVITVPAYFNDSQR.T	1580.1024	1579.7995
3.	R.QAVVNPENTFFSVKR.F	1735.2343	1734.9053
4.	K.KQDITITGASTLPQDEVER.M	2100.5066	2100.0699

Table 4C: The MALDI TOF mass spectrum sequence of peptides matched with gi|302786294 hypothetical protein VITISV_000728 of *Vitis vinifera* (Protein score: 118)

S. No.	Peptides identified by MS/MS	Mr (expt.)	Mr (calc.)
1.	K.DLDEVILVGGSTR.I	1372.9911	1372.7198
2.	K.QFAAEEISAQVLR.K	1461.0489	1460.7623
3.	R.QAVVNPENTFFSVKR.F	1735.2343	1734.9053
4.	K.SEVFSTAADGQTSVEINVLQGER.E	2436.6431	2436.1769

Mr: Nominal Mass

Table 4D: The MALDI TOF mass spectrum sequence of peptides matched with gi|302764140 hypothetical protein SELMODRAFT_230659 *Selaginella moellendorffii* (Protein score: 51)

S. No.	Peptides identified by MS/MS	Mr (expt.)	Mr (calc.)
1	K.LEFKDINEVILVGGSTR.I	1890.3833	1889.3760

Mr: Nominal Mass

control (Figure 2). Protein identification performed with the Mascot search engine in NCBI database indicated extensive homology of this protein with chloroplast heat shock protein 70 of *Cenchrus americanus* (Protein score: 295; Figure 3 and Table 4A), hypothetical proteins SELMODRAFT_267815 of *Selaginella moellendorffii* (Protein score: 125; Figure 3 and Table 4B) and VITISV_000728 of *Vitis vinifera* (Protein score: 118; Figure 3 and Table 4C) and SELMODRAFT_230659 of *Selaginella moellendorffii* (Protein score: 51; Figure 3 and Table 4D). High numbers of differently regulated proteins have been reported in roots and shoots of maize on treatment with NaCl (Zorb *et al.*, 2004).

Similarly, increase in protein content due to salinity stress has also been reported in bean plant (Amira and Qados, 2011). The analysis of plant proteome is an important alteration to the analysis of the genome, because gene expression is altered under salinity stress. Wahid *et al.* (2007) suggested that specific expression of stress proteins is an important adaptive manifestation in maintaining the integrity, native configuration and topology of cellular membrane components to ensure their normal functioning under salinity stress. In the present study, a significant negative correlation has been found between cell membrane stability and total protein content ($R = –0.32$).

The results reveal enhancement of RNA level in leaf segments at all the concentrations of NaCl (Figure 1B). It may be due to more requirement of transcription to cope with the adverse conditions. A significant correlation between the RNA and protein content of salt treated leaf segments is observed ($R = 0.74$). An increase in RNA content of wheat seedlings (Khan *et al.*, 1990) and *Brassica oleracea* (Mukhtar and Hasnain, 1994) has been reported under NaCl stress. Reduction in DNA content in leaf segments treated with 150-300 mM NaCl has been observed in the present study with R^2-value of 0.92 (Figure 1C). This may be due to direct degradation effect of NaCl on DNA or indirectly by the free radicals generated in the stressed leaf tissue because of oxidative damage. Nuclear and DNA degradation induced by salt stress has been demonstrated in meristematic cells of barley roots (Katsuhara and Kawasaki, 1996). Similarly Morozovskii and Kabanov (1968) suggested that protein and nucleic acid synthesis are most inhibited under chloride salinity.

5. Conclusion

The results demonstrated that the dark grown maize leaf segments exhibit a high degree of stress due to NaCl treatment which affects the overall growth and biochemical parameters governing the metabolic activities of the leaf tissue. Unchanged RWC of salt treated leaf segments indicates tolerance towards osmotic stress caused by salt. Thus, it seems that NaCl stress is mediated through ion toxicity, by disturbing the ionic status of leaf tissue. Further, it also seems that the stress induced proteins are being synthesized in leaf tissue in response to salt stress.

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