INT. J. BIOL. BIOTECH., 14 (2): 179-185, 2017.

PHYSIOLOGICAL AND BIOCHEMICAL ASSESSMENT OF SUGARCANE CALLI (CP 77/400) AGAINST VARIOUS LEVEL OF SALINITY STRESS

Saba Gul, Mohammad Sayyar Khan*, Mazhar Ullah, Waqar Ahmad, Syed Usman Ali Shah and Memrez Khushal

Institute of Biotechnology and Genetic Engineering (IBGE), The University of Agriculture Peshawar, Pakistan

*For Correspondence: sayyarkhankazi@aup.edu.pk Mobile Phone: +92-302-8347886

ABSTRACT

The present study was aimed to check the physiological and biochemical influence of NaCl stress on the calli of tissue culture responsive variety CP 77/400 *in vitro*. Four level of NaCl stress i.e. 50mM, 100mM, 150mM and 200mM was used in the experiment against the Control. The calli was exposed for 21 days (3 weeks) to different level of salinity and significant effects *($P \leq 0.05$)* on the physiological and biochemical properties of the cells were observed. Relative growth rate was significantly affected and was 0.28 g $week^{-1}$ at 200mM concentration as compared to other NaCl levels. Water content decreased as the level of salinity stress increased. At 200mM concentration water content was the lowest (66.000%) of all concentrations used. Electric Conductivity (EC) was higher at 200mM concentration (133.06 dSm^{-1}). Calli cells respond to NaCl stress by increasing the accumulation of proline and sugar content and both were statistically significant (*$P \leq 0.05$*). Proline accumulation was maximum at 200mM (36.748 µM g-1 FW) concentration in comparison to control. Similarly sugar accumulation increased significantly at 200mM NaCl Concentration (1.69481 µM g-1 FW).

Key Words: Salt Stress, CP 77/400, Physiological effects, Biochemical effects.

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.), a well-known and highly reputable member of the family *Poaceae* is grown worldwide to meet the sugar demand. Seventy percent of the world's sugar is harvested from the sugarcane crop (Lakshmanan *et al.,* 2005) while the remaining 30% is achieved from other sources- sugar beet etc. Sugarcane stores about 0.7 mole sugar in its internodes. In addition to sugar, sugarcane is also used for ethanol production. Sugarcane mud is used as a natural fertilizer (Lingle and Weigan, 2000). Currently cultivated sugarcane (*Saccharum officinarum L.*) is developed by interspecific cross of 5 different kinds of sugarcane species (Khan *et al.,* 2013). *Saccharum officinarum* L. has a chromosome number 10.

The increasing level of salinity is amongst the severe threats to sugarcane crop. Significant losses to sugarcane crop as a result of salt stresses have been reported. Sugarcane yield is reduced to more than 50% as result of salinity stress (Wiedenfeld, 2008). Reactive Oxygen Species (ROS) is the metabolic agitation due to osmotic effects of salt or ionic toxicity of salt stress or dehydration stress (Neill *et al.,* 2002; ImLay, 2003). Pakistan is ranked fourth with respect to cultivating area of sugarcane however still its cane production is smaller as compared to other sugarcane producing countries (Mian and Saeeda, 2003). In Pakistan low cane yield is also associated with the increasing level of salinity in the cultivated land (Akhtar *et al.,* 2003).

Plant adopts certain strategies to compete with stresses. In order to develop effective strategies against salinity stresses a comprehensive study about sugarcane physiology and biochemistry is necessary. Sugarcane genetic transformation with gene conferring salt tolerance is amongst the strategies however successful transgenic approaches depends on the effective and productive tissue culture protocols for the crop. Using tissue culture *in vitro* mutants for a crop can be developed and also the already available varieties can be screened for salt tolerance.

In vitro techniques offers control environment for studying Physiological and biochemical effects of salt stresses both at cellular (Ahmad *et al*, 2007) and plant level (Lokhande *et al.,* 2011; Kumar *et al.,* 1994; Wahid *et al.,* 1997). The present study is aimed to screen the tissue culture responsive CP 77/400 variety for NaCl stress.

MATERIAL AND METHODS

The research was conducted at Genomics and Bioinformatics Lab (GBL), Institute of Biotechnology and Genetic Engineering (IBGE), The University of Agriculture, Peshawar.

Sugarcane Genotypes

CP-77/400 was collected from the Sugarcane Crop Research Institute (SCRI), Mardan, Pakistan were used in the experiment. The present research was facilitated by Institute of Biotechnology and Genetic Engineering, The University of Agriculture Peshawar.

Callus Induction Media Preparation

MS basal salt media (Murashige and Skoog, 1962) having 5mg L^{-1} 2,4.D and 5% Coconut water (Ullah *et al.*, 2016), solidified with 6-7% agar (pH 5.8) was used for callus induction. Media was sterilized through autoclave and poured in petri plates.

Sterilization of Explant

Healthy young meristems were collected from sugarcane variety (CP 77400) and were thoroughly washed with tape water. The explant were than rinsed thoroughly with 70 % ethanol for 5 minutes followed by washing with distilled water for 3 to 5 times in a laminar flow hood. Finally, the explants were dried on sterilized filter papers before the inoculation on to sterilized MS media supplemented with 10% CW+5mgL^{-1} 2,4-D as previously reported by (Ullah *et al.*, 2016).

Environmental Conditions

The callus cultures were kept in dark for one week and were then exposed to light. The temperature of the growth room was maintained at 28 ± 2^{o}C with 70-80% relative humidity in the culture room.

***In vitro* Salt Stress**

The induced calli after four sub cultures were cultured on salt stress callus induction media having different concentration of NaCl (50mM, 100mM, 150mM and 200mM). The treated calli were exposed to salt stress against the control media for a week and the growth was observed regularly.

Parameters

Relative Growth Rate (RGR)

RGR was estimated after 21 days of the culture according to the method of Amin *et al.*, (2013), Shah *et al.* (1990) and Patade *et al.* (2012). Petri plates with MS media were weighed before and after inoculation of the callus to estimate the initial weight of the callus. The callus was then incubated at 28±2 ^{o}C for 3 weeks. The relative growth rate was calculated by the formula;

RGR week^{-1} = (fresh weight (FW)-initial FW)/3

Water Content

Fresh callus of weight 500 mg was taken and placed in oven for 50 hours at 75 ^{o}C. Dried callus was again weighed. Water content (WC) was determined according to (Patade *et al.*, 2012) formula;

WC% = [(FW – DW)/ FW] × 100

Electric Conductivity

Electrolytic conductivity of diluted sap was measured to check the increase in ionic concentration of the cells. Homogenized tissues (200 mg) were centrifuged at 10,000 rpm for 12 min. The supernatant (cell sap) was collected and the volume was adjusted to 10 mL by adding deionized water. Electrical conductivity was then measured in (dSm^{-1}) by Electric Conductivity Meter (ECM).

Proline and Sugar Analysis

Callus was weighted 500 mg and crushed using mortar and pastel. Sample was added to test tubes along with 5mL Methanol: Chloroform: H_2O in ratio of 12:5:1. The Sample was centrifuged for 5 minutes at 5000 rpm. Supernatant was transferred to other test tube. Then with pressure 2mL chloroform and 3 mL Distilled water ($D.H_2O$) was poured and shaken well.

Proline Estimation

Accumulated proline was calculated according to the procedure of (Bates *et al.*, 1973). In laminar air flow hood 0.5 mL sample was taken and mixed 0.5 mL of methanol: $D.H_2O$ along with the addition of 1mL acetic acid. Sample was heated for 45 min at 100^{0}C after addition of 1mL ninhydrin solution. After cooling 5 mL toluene was

added. Upper layer of sample was evaluated at 520 nm with UV-visible spectrophotometer and absorbance was measured under a blank (toluene).

Estimation of Total Soluble Sugars

Total sugars were determined by the method of DuBois *et al.* (1956). 1mL dH_2O with 1mL sample was taken in a test tube, after which 5mL sulphuric acid and 1mL phenol was mixed with sample. All the test tubes were then placed in the fume hood. Test tubes were cooled at room temperature and placed on the shaker for 12 minutes. Total soluble sugar content was calculated at 490nm against D-glucose (blank) using Spectrometer.

Statistical analysis

Collected data was subjected to the analysis of variation and least significance difference by using statistix 8.1.

RESULTS

Callus induction

Already optimized callus induction medium $5mgL^{-1}$ 2.4,D+10% coconut water by was used for CP 77/400 variety. Previously CP 77/400 have been analyzed for its tissue culture capacities and has been recommended for sugar transgenic programs. Similar efficient callogenesis was observed (80%) as reported previously (as shown Figure). Based on its capabilities, CP 77/400 was selected for *in vitro* screening of salinity stress.

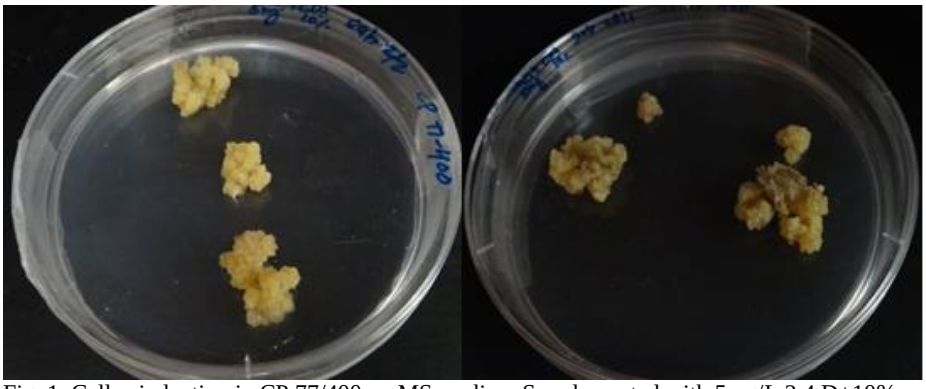

Fig. 1. Callus induction in CP 77/400 on MS medium Supplemented with 5mg/L 2.4,D+10% coconut water.

Salt stress analysis

Effects on relative growth rate (RGR)

Reduction in growth was observed when calli were subjected to increasing level of salt stress (Table 1). ANOVA suggested significant differences *($P \leq 0.05$)* in RGR at different concentration of NaCl. Maximum RGR i.e 1.19 g $week^{-1}$ was observed at control followed by 0.94 g $week^{-1}$ at 50mM concentration. Lowest was observed at 200mM i.e 0.28 g $weeks^{-1}$ concentration of NaCl.

Table 1. Relative Growth Rate (RGR) and Water content of CP 77/400 calli after 20 days of NaCl stress. Values are means ± S.D.

Salt Treatments	RGR (g $week^{-1}$)	Water Content (%)
Control	$1.19^{a} \pm 0.08$	$90.55^{a} \pm 1.81$
50mM	$0.94^{b} \pm 0.04$	$88.66^{a} \pm 0.34$
100mM	$0.74^{c} \pm 0.09$	$85.33^{b} \pm 1.73$
150mM	$0.48^{d} \pm 0.05$	$74.88^{c} \pm 1.28$
200mM	$0.28^{e} \pm 0.09$	$66.00^{d} \pm 1.00$

Mean values in the column having different letters are significantly ($P \leq 0.05$) different from each other using LSD; The ± represents standard deviation.

Effects on water content

Water content of calli was affected significantly *($P \leq 0.05$)* upon exposure to different level of salt stresses (Table 2). However at Control and 50mM salt concentration no statistically significant ($P \geq 0.05$) decrease in water

content has been observed. Maximum water content was observed at Control i.e 90.55% followed by 88.66% at 50mM concentrations. While at 200mM, lower water content has been observed i.e 66.00%. Overall salt stress affected the water content of the calli depending upon the concentration of the salt.

Electric Conductivity (EC)

ANOVA suggested significant differences *(P≤ 0.05)* in electric conductivity of cell cap at different concentrations of NaCl (Table 8). Low EC (60.344 dSm^{-1}) has been observed at control followed by 77.789 dSm^{-1} at 50mM. . Maximum EC has been observed when NaCl concentration of 200mM was used in callus induction media.

Table 2. Electric conductivity (EC) of CP 77/400 calli after 20 days of NaCl stress. Values are means ± S.D.

Treatments	Electric Conductivity (dSm-1)
Control	60.34^{e} ± 1.19
50mM	77.78^{d} ± 1.17
100mM	95.23^{c} ± 1.83
150mM	108.01^{b} ± 1.32
200mM	133.06^{a} ± 1.55

Mean values in the column having different letters are significantly (*P≤ 0.05*) different from each other using LSD; The ± represents standard deviation.

Accumulation of Free Proline

Plants accumulate proline in order to compete with the salt stresses which results adverse effects on its growth condition and viability of the cells. At control, 50mM and 100mM the differences in proline accumulation was statistically no significant (*P≥ 0.05*) showing 14.51, 16.34 and 17.40 µM g^{-1} FW accumulation frequencies respectively. However proline accumulation at 150mM (21.40 µM g^{-1} FW) and 200mM (36.74 µM g^{-1} FW) showed significant differences *(P≤ 0.05)* from each other as well as with Control, 50mM and 100mM NaCl concentration used. Lower proline accumulation was observed at control i.e 21.40 µM g^{-1} FW followed by 50mM concentration (16.34 µM g^{-1} FW) while maximum accumulation of 36.74 was observed at 200mM concentration (Table 3).

Accumulation of Total Soluble Sugar

An Increase in sugar accumulation has also been observed however the degree of increase was small as compared to proline content where the increase was intensive (Table 4). Sugar accumulation at 50mM and 100mM was statistically non-significant (*P≥ 0.05*) from each other. Sugar accumulation at 150mM concentration showed non-significant (*P≥ 0.05*) differences with 50mM and 200mM NaCl concentration (Table 4). Lower sugar accumulation was observed at control i.e 1.14 µM g^{-1} FW followed by 1.39 µM g^{-1} FW at 50mM Nacl Concentration while maximum sugar accumulation (1.69 µM g^{-1} FW) was observed at 200mM NaCl concentration (Table 4) suggesting that an increase on NaCl concentration results in higher sugar accumulation.

Table 3. Total soluble sugar and free proline content of 20 days salt stressed CP 77/400 calli. Values are means ± S.D.

Treatments	Sugar contents (µM g^{-1} FW)	Proline Content (µM g^{-1} FW)
Control	1.14^{d} ± 0.20	14.51^{c} ± 0.01
50Mm	1.39^{c} ± 0.13	16.34^{bc} ± 1.30
100mM	1.51^{bc} ± 0.11	17.40^{bc} ± 1.35
150Mm	1.60^{ab} ± 0.09	21.40^{b} ± 1.45
200Mm	1.69^{a} ± 0.02	36.74^{a} ± 2.35

Mean values in the column having different letters are significantly *(P≤ 0.05)* different from each other using LSD; The ± represents standard deviation.

DISCUSSION

Tissue culture science from the last few decades has emerged as an indispensible technology to meet the increasing demands of disease free germplasm through micropropogation (Vasil *et al.,* 1982). In addition it is practiced to develop *in vtiro* mutants for various biotic and abiotic factors. Tissue culture technologies are exploited for the *in vitro* screening of salt tolerant crops a quick and efficient technology in comparison with formal practices for the development and conservation of germplasm of best varieties of sugarcane. The present study was aimed to

screen the tissue culture responsive variety CP 77/400 physiologically and biochemically for its tolerance to salt stresses by subjecting it to various level of salt stresses.

Enormous physiological and biochemical impact in response to increasing level of NaCl was observed after 21 days of exposure to stress. Salt stress enhanced the electric conductivity of cell sap due to increase level of ion concentrations which results an adverse effect on the growth and osmotic pressure. Increase in ionic content due to salt stress was also reported (Amin *et al.,* 2013). The presents study reveals hyper ionic content in response to increasing level of NaCl and low relative growth rate (RGR). Similar results were reported previously (Tester and Davenport 2003) which confirmed both ionic and osmotic stresses due higher concentration of Na^+ and Cl^- ions. Metabolic imbalances and ionic toxicity causing destruction of cellular growth in late phase due to salt stresses has also been reported by (Munns and Tester, 2008). This refers metabolic imbalances and ionic stresses being salt stress induced. Sugarcane being a hard crop competes with the salinity stresses but beyond certain limits it loses its competences against salt stresses and its growth is affected adversely. These results about growth retardation and sensitivity of undifferentiated, actively growing tissue in response to increasing level salinity stresses are in parallel to the results revealed by Errabii *et al.* (2007). Cell utilizes much of its energy to counter salt stress which results in a depletion of energy for the normal growth of the cells (Cushman *et al.,* 1990). Similar growth retardation in sugarcane was also observed (Nasir *et al.,* 2000) in response to NaCl stress which confirms the results of the present study. The present study also reveals the gradual decline in water content due to increasing level of NaCl stress which supports the results (Ahmad *et al.,* 2007) suggesting water loss as an outcome of salt stress. In comparison to growth the decrease in water retention was not high which suggest that plant cells have active osmotic adjustment mechanism for overcoming water loss due to salt stresses. Patade *et al.* (2012) also reported the efficiency of cells in maintaining the water content. Exposure to NaCl stress results in higher accumulation of Na^+ and Cl^- which results in hyper electric conductivity. Electric conductivity was elevated upon increasing the NaCl concentration in the growth media. An increase in electric conductivity due to accumulation of high Na^+ and Cl^- ions was also reported by Karpe *et al.* (2012).

Accumulation of free proline is amongst the common strategies of the cells to compete with the salt stresses. Building up free proline in response to salt stresses provides protection to cytoplasm against dehydration Proteins degradation inside cells occurs in salt stresses which results in the accumulation of free proline (saied *et al.,* 2009). The present study confirms the accumulation of free proline in the cells in response to NaCl stress. Shah *et al.* (1990) and Patnaik and Debata (1997) also reported the accumulation of free proline due to salt stresses. Protecting cytoplasmic dehydration by accumulating free proline was also reported by Hasegawa *et al.* (1986) which confirmed our results of free proline accumulation in response to stress. Cells compromise its growth and utilize its resources on accumulation of free proline which results retardation in growth. Associating growth retardation with free proline accumulation due to salt stress is also reported by Shah *et al.* (1990) and Cano *et al.* (1996).

Sugar is associated with numerous biochemical processes such as respiration, photosynthesis and translocation. Under salt stress condition, plant reduces its osmotic potential in order to enhance water uptake by accumulating mineral ions and synthesizes sociable solutes as water deficiency or drought stress effect. The present study confirmed the increase of sugar content in response to NaCl stress. Similar results were previously reported by Munns and Weir, (1981). However the reports of Morgan, (1922) contradicted the increase of sugar accumulation as a result of NaCl stress. Kerepsi and Galiba (2000) confirmed the present result and contradicted (Morgon, 1992) for sugar accumulation being not affected as result of salt stress.

Conclusion

Based on *in vitro* screening of CP 77/400 for salt stress analysis it is clear that salinity influences both physiological and biochemical processes of the cell. Cells upon exposure to salinity stress consume its energy on competing against the stress and compromise its growth. As a result retardation in growth occurs. Cells also direct the accumulation of sugar and free proline for its survival and to avoid destruction caused by salinity stress.

Acknowledgment

Pakistan Science Foundation (PSF) is highly acknowledged for funding the project entitled *"Genetic engineering of sugarcane With Rice Tonoplast H+-ppase gene to improve sucrose content and salinity tolerance"*. Institute of Biotechnology and Genetic Engineering (IBGE) is also highly acknowledged for facilitating this project.

REFERENCES

Ahmad, M. S. A., F. Javed and M. Ashraf (2007). Iso-osmotic effect of NaCl and PEG on growth, cations and free proline accumulation in callus tissue of two indica rice Oryza sativa L. Genotypes. *Plant Growth Regul*., 53:53–63.

Akhtar, S., A. Wahid and E. Rasul (2003). Emergence, growth and nutrient composition of sugarcane sprouts under NaCl salinity. *Biol. Plant*., 46: 113-116.

Amin, N. U., G Sanga, N. Ara, S. H. Shah and F. Ullah (2013). Effect of various concentrations of calcium chloride on callus growth and potassium nutrition of calli cultures of potato (solanum tuberosum). *Pak. J. Bot*., 45(1): 209-214.

Bates, L.S., R.P. Waldren and I.D. Teare (1973). Rapid determination of free proline for water stress studies. *Plant Soil*, 39: 205-207.

Cano, E. A., F. Perez-Alfocea, V. Moreno, M. Caro and M. C Bolarin (1996). Responses to NaCl stress of cultivated wild tomato species and their hybrids in callus cultures. *Plant Cell Rep*., 15:791-794.

Cushman, J. C., E. J. De-Rocher and H. J. Bohnert (1990). Gene expression during adaptation to salt stress. In: Environmental Injury to Plants (ed. F. Katterman). Academic Press, Inc. Mechanisms of salinity tolerance. *Ann. Rev. Plant Biol*., 59:651–681.

DuBois. M., K. A. Gilles, J. K. Hamilton, P. A. Rebers and Fred. Smith (1956). Colorimetric Method for Determination of Sugars and Related Substances. *Anal. Chem.*, 28 (3), 350–356.

Errabii, T., C. B. Gandonou, H. Essalmani, J. Abrini, M. Idaomar and N. S. Senhaji (2007). Effects of NaCl and mannitol induced stress on sugarcane Saccharum sp. callus cultures. *Acta Physiol. Plant*, 29:95–102.

Hasegawa, P. M., R. A. Bressan and A. K. Handa (1986). Cellular mechanisms of salinity tolerance. *Hort. Science*, 21:1317-1324.

ImLay, J. A. (2003). Pathways of oxidative damage. *Annu. Rev. Microbiol*., 57: 395–418.

Karpe, A., A.A. Nikam, K.P. Chimote, S.B. Kalwade, P.G. Kawar, H. Babu, R.M. Devarumath and P. Suprasanna (2012). Differential responses to salinity stress of two varieties (CoC 671 and Co 86032) of sugarcane (*Saccharum Officinarum* L.). *Afr. J. Biotechnol*., 11(37):9028-9035.

Kerepsi, I. and G. Galiba (2000). Osmotic and salt stress induced alternation in soluble carbohydrate content in wheat seedling. *Crop Sci*., 40: 482–487.

Khan, S.A., Z. Hanif, U. Irshad, R. Ahmad, M. Yasin, M.F. Chaudhary, A. Afroz, M.T. Javed, U. Rashid and H. Rashid (2013). Genetic transformation of sugarcane variety HSF-240 with marker gene GUS. *Int. J. Agric. Biol*., 15(6): 1258–1264.

Kumar, S., K. M. Naidu and H. J. Sehtia.(1994). Causes of growth reduction in elongating and expanding leaf tissue of sugarcane under saline conditions. *Aust. J. Plant Physiol*., 21:79–83.

Lakshmanan, P., R. J. Geijskes, K. S. Aitken, C. L. P. Grof, G. D. Bonnett and G. R. Smith (2005). Sugarcane biotechnology: the challenges and opportunities. *In vitro. Cell .Dev. Biol. Plant*. 41:345–363.

Lingle, S. E. and C. L. Weigand (1996). Growth and yield responses of sugarcane: II. Sucrose biochemistry in individual internodes. Proc. Inter-American Sugarcane Seminars. pp. 93-102.

Lokhande, V. H., T. D. Nikam, V. Y. Patade, M. L. Ahire and P. Suprasanna (2011). Effects of optimal and supra-optimal salinity stress on antioxidative defence, osmolytes and *in vitro* growth responses in *Sesuvium portulacastrum* L. *Plant Cell Tiss. Org*., 104:41–49.

Mian, M.N. and Saeeda. (2003). *Farm productivity size and tenure and estimation of domestic resource cost for major crops in NWFP*. Center for Appl. Econ. Studies, The University of Peshawar, Pakistan 106-116.

Morgan, J. M. (1992). Osmotic components and properties associated with genotypic differences in osmoregulation in wheat. *Aus. J. Plant Physiol*., 19: 67–76.

Munns R. and R. Weir (1981). Contribution of sugars to osmotic adjustment in elongating and expanded zones of wheat leaves during moderate water deficit at two light levels. *Aus. J. Plant Physiol*., 8: 93–105.

Munns, R. and M. Tester (2008). Mechanisms of salinity tolerance. *Annu. Rev. Plant Biol*., 59: 651-81.

Murashige, T. and F. Skoog (1962). A revised media for rapid growth and bioassay with tobacco tissue culture. *Physiol. Plant*, 15: 473-497.

Nasir, M. N., R. H. Qureshi, M. Aslam and J. Akhtar (2000). Screening of sugarcane lines selected through hydroponic studies in naturally salt affected field. *Pak. Sugar J*., 15: 2- 10.

Neill, S., R. Desikan and J. Hancock (2002). Hydrogen peroxide signaling. *CurrOpin Plant Biol*., 5:388–395.

Patade, V. S., B. Sujata and S. Penna (2012). Effects of NaCl and isoosmotic PEG stress on growth, osmolytes accumulation and antioxidant defense in cultured sugarcane cells. *Plant Cell Tiss. Org*., 108: 279-286.

Patnaik, J. and B. K. Debata (1997). *In vitro* selection of NaCl tolerant callus lines of *Cymbopogon martinii* (Roxb.) Wats. *Plant Sci*., 124:203-210.

Saeid, M., A. Romman and M. A. Suwwan (2009). Salt Stress-Induced Responses in Cucumber Callus. Dirasat, *Agri. Sci*., 36(2): 101-107.

Shah, S. H., S. J. Wainwright and M. J. Merrett (1990). The interaction of sodium and calcium chlorides and light on growth, potassium nutrition and proline accumulation in callus cultures of *Medicago sativa* L. *New Phytol*., 116:37-45.

Tester, N. and R. Davenport **(**2003). Na^+ tolerance and Na^+ transport in higher plants. *Annals. of Botany*, 91**:** 1–25.

Ullah. M., H. Khan, M. S. Khan, A. Jan, K. Ahmad and A. W. Khan (2016). *In vitro* Plant Regeneration of Sugarcane (S*accharum officinarum* l.); The Influence of Variety, Explant, Explant position and Growth Regulators. *ARPN J. Agric. & Bio. Sci*., 11 (7): 267-273.

Vasil, I. K., W.R. Scowcroft and K. J. Frey (1982). *Plant Improvement and Somatic Cell Genetic.*, Academic Press, NY., 158-178.

Wahid, A., A. R. Rao and R. Ejaz (1997). Identification of salt tolerance traits in sugarcane lines. *Field Crop Res*., 541:9–17.

Wiedenfeld, B. (2008). Effects of irrigation water salinity and electrostatic water treatment for sugarcane production. *Agric.Water Manag*., 95:85–88.

(*Accepted for publication February 2017*)