Acta Physiol Plant (2007) 29:95–102
DOI 10.1007/s11738-006-0006-1

ORIGINAL PAPER

Effects of NaCl and mannitol induced stress on sugarcane (*Saccharum* sp.) callus cultures

Tomader Errabii · Christophe Bernard Gandonou · Hayat Essalmani · Jamal Abrini · Mohamed Idaomar · Nadia Skali Senhaji

Received: 11 December 2005 / Accepted: 4 May 2006 / Published online: 25 January 2007

Abstract The effects of NaCl and mannitol iso-osmotic stresses on calli issued from sugarcane cultivars (cvs.) R570, CP59-73 and NCo310 were investigated in relation to callus growth, water content, ion and proline concentrations. Callus growth and water content decreased under both stresses with the highest reduction under mannitol-induced osmotic stress. The ion concentration was drastically affected after exposure to NaCl and mannitol. Salt stress induced an increase in Na^+ and Cl^- accumulation and a decrease in K^+ and Ca^{2+} concentrations. Under mannitol-induced osmotic stress, K^+ and Ca^{2+} concentrations decreased significantly while Na^+ and Cl^- concentrations remained unchanged. Free proline accumulation occurred under both stresses and was more marked in stress-sensitive cv. than in stress-resistant one. Our results indicated that the physiological mechanisms operating at the plant cell level in response to salt- and osmotic-induced stress in sugarcane cvs. are different. Among the cvs., we concluded that the stress resistance is closely related to the maintain of an adequate water status and a high level of K^+ and Ca^{2+} under both stresses and a low level of Na^+ concentration in the presence of NaCl. Thus, sugarcane (*Saccharum* sp.) can be regarded as a Na^+ excluder. We also provided evidence that proline accumulation is a stress-sensitive trait rather than a stress resistance marker.

Keywords Sugarcane (*Saccharum* sp.) · NaCl · Mannitol · Callus growth · Ion uptake · Proline

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
cv(s)	Cultivars
MS	Murashige and Skoog
RGR	Relative growth rate
ANOVA	Analyses of variance

Introduction

Salt stress has been extensively investigated since soil salinity represents a major constraint for successful production and crop yielding (Munns et al. 2002). The salt-affected lands extended to about 6% of the world surface and are becoming even more prevalent as the intensity of agriculture increases worldwide (Flowers and Yeo 1995). Thus, a large percentage of agricultural lands are naturally saline or are salinized by inadequate irrigation practices (Chinnusamy and Zhu 2003). High-salt stress disrupts homeostasis in water potential and ion distribution at both the cellular and the whole plant levels (Zhu 2001; Munns et al. 2002). Excess of Na^+ and Cl^- ions may lead to conformational changes in protein structures, while the osmotic stress leads to turgor loss and cell volume change (Chinnusamy

Communicated by M. Filek.

T. Errabii · C. B. Gandonou · J. Abrini · M. Idaomar · N. Skali Senhaji (✉)
Laboratoire de Biologie et Santé, Equipe de Biotechnologies et Microbiologie Appliquée, Université Abdelmalek Essaâdi, Faculté des Sciences, BP 2121 Tétouan, Morocco
e-mail: skali@fst.ac.ma

H. Essalmani
Laboratoire de Biotechnologie Végétale, Université Abdelmalek Essaâdi, Faculté des Sciences et Techniques, BP 416 Tanger, Morocco

and Zhu 2003). However, the precise mechanisms underlying these effects are not fully understood because the resistance to salt stress is a multigenic trait (Parida and Das 2005). To achieve salt tolerance, plant cells evolve several biochemical and physiological pathways. These processes are thought to operate additively to ensure plants and cells survival and they include the exclusion of Na^+ ions and their compartmentation into vacuoles as well as the accumulation of compatible solutes such as proline, glycinebetaine and polyols (Hasegawa et al. 2000; Chinnusamy and Zhu 2003; Parida and Das 2005).

Sugarcane is a glycophyte crop of major economical value in tropical and subtropical developing countries where salinity is an ever-increasing problem (Wahid et al. 1997). In Morocco, sugarcane is grown under irrigated systems and is seriously prone to soil salinization. This problem may be a serious handicap for the production and the yielding of this agricultural crop.

In vitro tissue culture constitutes an important tool to study the physiological and biochemical mechanisms that operate in response to stress conditions at the cellular level (Lerner 1985). Furthermore, the plant tissue culture allows the control of stress homogeneity and the characterization of cell behaviour under stress conditions, independently of regulatory systems that take place at the whole plant level (Lutts et al. 2004).

The present work was performed using three sugarcane (*Saccharum* sp.) cultivars (cvs.), R570, CP59-73 and NCo310, differing in their salt resistance at the whole plant level with the objective to investigate the effects of the osmotic and the ionic component of salt stress by the addition of ionic (NaCl) and non-ionic (mannitol) agents to the culture medium at iso-osmotic concentrations at the plant cell level. The evolution of the calli growth in relation to water status, ion and proline accumulation under both stresses was discussed.

Materials and methods

Plant material and culture conditions

The three sugarcane (*Saccharum* sp.) cvs. R570, CP59-73 and NCo310 were kindly provided by CTCS (Centre Technique des Cultures Sucrières du Gharb, Kenitra, Morocco). Stem sections of the three cvs. containing two lateral buds were planted in plastic pots containing soil and they were grown under greenhouse conditions until reaching ~6 months. The cvs. used in this work have been characterized for their salt resistance at the whole plant level (unpublished data). NCo310 (Natal Coimbatore, South Africa), is a salt-resistant cv. R570 (CERF, Sainte-Clotilde Cedex, Reunion) is a leading commercial variety in several countries and is a salt-sensitive cv., while CP59-73 (Canal Point, FL, USA) is largely cultivated in Morocco and it behaves as an intermediary salt-resistant cv.

Calli were induced from leaf segments of the youngest leaves. They were surface sterilized with 75% (v/v) ethanol. Then, they were immersed in mercuric chloride $HgCl_2$ 0.03% (w/v) for 30 min followed by three rinses with sterile distilled water for 10 min each. Explants were aseptically inoculated onto Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 2 mg l^- 2,4-D. The pH value was adjusted to 5.8. Callus induction was performed in the dark at 25 ± 2°C in a growth chamber.

Growth and water content determination

After 6 weeks, calli were individually weighed and placed on MS medium supplemented or not with the stress factors. Iso-osmotic concentrations of NaCl (50, 100 and 150 mM) and mannitol (100, 200 and 300 mM) were applied. For each treatment (cv. × stress factor × concentration), 30–35 Calli were used. After 4 weeks of the exposure to stress conditions, the calli were weighed and then they were characterized for ion and proline concentrations.

Callus relative growth rate (RGR) was determined on a fresh weight (FW) basis according to the formula; $RGR = [(FW_f - FW_i)/FW_i]$, where FW_f and FW_i are the final and initial FW of the calli. The callus water content was calculated using the formula; [(FW – DW)/DW], where FW and DW are, respectively, the fresh weight and dry weight of the calli.

Determination of ion concentration

For ion measurements, calli were first rinsed for 5 min with cool distilled water in order to remove free ions from the apoplasm without substantial elimination of cytosolic solutes as recommended by Sacchi et al. (1995). Calli were oven-dried at 80°C for 72 h and they were then grounded. The dry matter obtained was used for mineral analysis. The major cations were extracted after digestion of dry matter with HNO_3 acid according to Lutts et al. (1996a). The extract was filtered prior to analysis. Na^+ and K^+ concentrations were determined using a flame spectrophotometer (PHF 90D, France). Ca^{2+} concentration was quantified by atomic absorption spectrophotometer (Shimadzu AA-6200, Kyoto,

Japan). For Cl^- content estimation, ions were extracted with hot distilled water (80°C during 2 h). Chloride was determined spectrophotometrically at 470 nm with ferric ammonium sulphate and mercuric thiocyanate as described by Guerrier and Patolia (1989).

Determination of proline concentration

Proline was extracted as described by Paquin and Lechasseur (1979). About 200 mg of callus fresh matter was homogenized in 4 ml of methanol–chloroform–water (15:5:1 v/v/v) at 4°C and then was centrifuged at 0°C and centrifuged at 20,000*g* for 30 min. Supernatants were then incubated at 4°C for 12 h in presence of 0.25 ml chloroform and 0.9 ml distilled water. Proline was quantified in the upper phase using ninhydrin acid reagent according to Bates et al. (1973). The chromophore containing proline was extracted in 4 ml of toluene and measured spectrophotometrically at 520 nm. L-proline was used as standard.

Statistical analysis

The experiments were repeated two times and gave similar trends. Data presented hereafter are from the two experiments. Each value is presented in the form of mean ± standard error with a reading of six independent samples per treatment. The analyses of the main effects of the stress factor, the cv., and the applied concentrations were based on a three-way analysis of variance at 5% level (SAS Institute Inc. 1988). When the main effect was significant, differences between means were evaluated for significance by using the least significant difference method (LSD). For each treatment, the data related the Na^+ and Cl^- were subjected to variance analysis (ANOVA II) at 5% level of confidence, considering the cvs. and the NaCl concentration as variable.

Results

Callus growth and water content

NaCl- and mannitol-induced stress decreased RGR among all the cvs. However, a highly significant difference was recorded among the effects of each kind of stress (Table 1). Mannitol-induced osmotic stress seemed to be more harmful to CP59-73 and NCo310 callus RGR than NaCl-induced stress. In contrast, callus RGR of R570 decreased more under salt-induced stress than mannitol-induced osmotic stress. A differential effect was also recorded among the cvs. RGR reduction corresponded to 65, 50 and 49% of the control at the highest concentration of NaCl and to 60, 63 and 58% of the control in the presence of mannitol iso-osmotic concentration in R570, CP59-73 and NCo310, respectively (Fig. 1).

The decrease of callus water content was observed under both NaCl- and mannitol-induced stress. However, a significant difference was recorded among the effects triggered by each kind of stress (Table 1). The highest reduction was noticed under mannitol-induced osmotic stress at all the experimented doses and it reached to about 45, 48 and 42% of the control in the presence of the highest mannitol concentration while it to about 25, 32 and 24% of the control in the presence of NaCl iso-osmotic concentration in R570, CP59-73 and NCo310, respectively (Fig. 2). Thus, the water content followed the same tendency as the callus RGR among the cvs.

Ion concentration

In the absence of stress, Na^+ concentration differed significantly ($P < 0.001$) among the cvs., and was lower in salt-sensitive than in salt-resistant cv., while no significant difference was recorded among cvs. in relation to K^+ and Cl^- concentrations. A highly significant difference was recorded among the effects of mannitol and NaCl in relation to the callus ion concentration. The exposure to NaCl induced an increase in Na^+ (Fig. 3) and Cl^- (Fig. 4) concentrations as well as a decrease in K^+ concentrations (Fig. 5). Cl^- concentration increased to about 340, 368 and 411% of the control in R570, CP59-73 and NCo310, respectively, at the highest NaCl concentration. Thus, the salt resistant NCo310 accumulated more Cl^- than salt sensitive R570. In contrast, the accumulation of Na^+ under salt-induced stress and the leakage of K^+ under both stresses were greater in the stress-sensitive than in stress-resistant cv. We observed, at the highest NaCl concentration, that Na^+ concentration increased to about 699, 394 and 375% of the control in R570, CP59-73 and NCo310, respectively. While, K^+ concentration decreased to 50, 43 and 38% of the control at the same NaCl concentration (Fig. 5) while it decreased to about 31, 48 and 32% of the control in the presence of mannitol iso-osmotic concentration in R570, CP59-73 and NCo310, respectively.

In contrast, mannitol-induced osmotic stress did not affect the Na^+ (Fig. 3) and Cl^- (Fig. 4) concentrations regardless of the mannitol concentration in the medium, while a decrease of K^+ concentration among the cvs. was observed (Fig. 5). A highly significant

Table 1 Results of three-ways variance analyses for RGR, callus water content, ion and proline concentrations of sugarcane calli; *F*-ratios are given for the main effects of the following levels of classification: stress factor (mannitol or NaCl), Cv. (cultivars) and stress intensity (i.e. concentration of stress factor in the medium) and interaction between the stress factor and the cultivar

Parameter	Stress factor	Cv.	Stress intensity	Interaction factor × cv.
Callus growth rate	21.51**	52.48**	84.14**	21.75**
Callus water content	331.4**	375.17**	99.68**	21.69**
K^+	237.7**	8.64**	131.83**	45.86**
Ca^{2+}	217.94**	5.77*	164.43**	22.86**
Proline	20.21**	51.91**	83.39**	20.63**

*$P < 0.01$; **$P < 0.001$

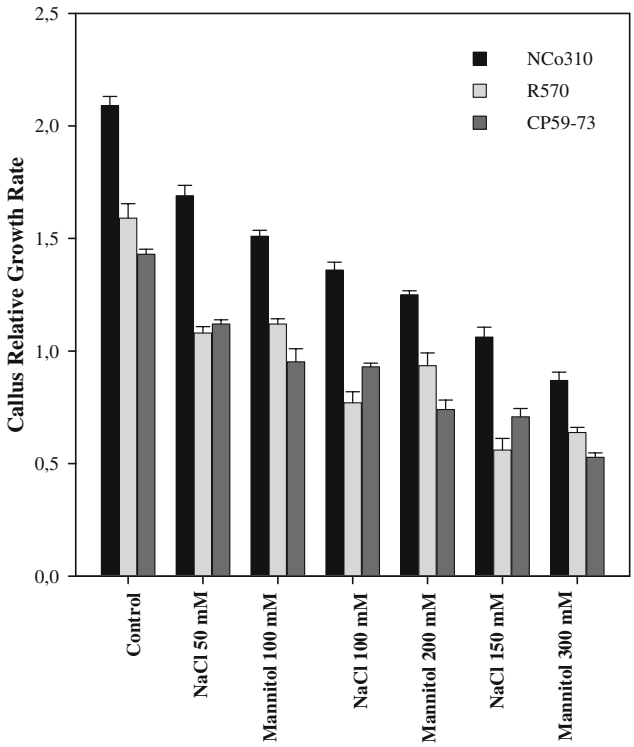

Fig. 1 Effect of NaCl and mannitol induced stress on callus relative rate growth of sugarcane (*Saccharum* sp.) cvs. NCo310, R570 and CP59-73 calli after 4 weeks of exposure to stress. Each value is the mean of six replicates and *vertical bars* represent ±standard error

difference was recorded among the effects of salt- and mannitol- induced stress for the Ca^{2+} concentration (Table 1). Ca^{2+} reduction was more marked under salt stress and it reached to about 57, 62 and 37% of the control in the presence of the highest NaCl concentration of NaCl and to about 32, 50 and 28% of the

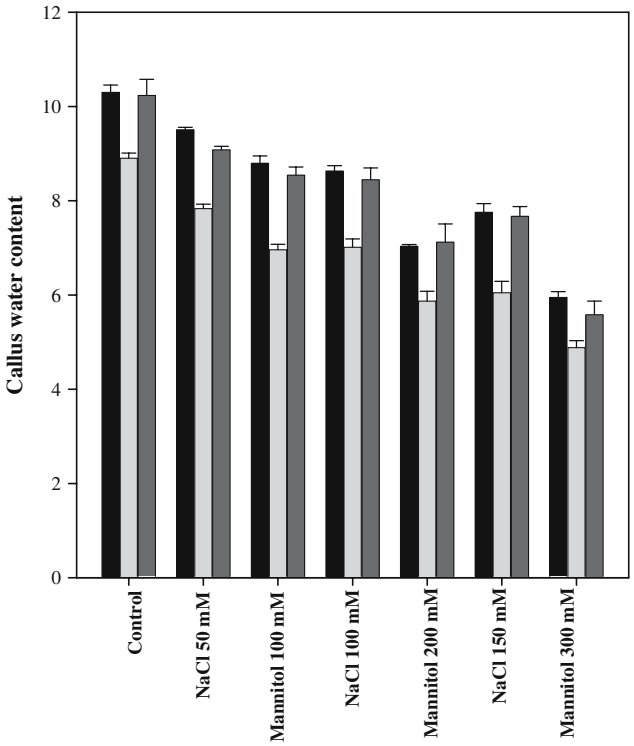

Fig. 2 Changes in callus water content of sugarcane (*Saccharum* sp.) cvs. NCo310, R570 and CP59-73 calli as affected by NaCl and mannitol induced stress after 4 weeks of exposure to stress. Each value is the mean of six replicates and *vertical bars* represent ±standard error

control in R570, CP59-73 and NCo310, respectively, in the presence of mannitol iso-osmotic concentration (Fig. 6). Among the cvs., we observed that the stress-resistant cv. maintained a higher Ca^{2+} concentration than the stress-sensitive one under both salt- and mannitol-induced stress.

Free proline accumulation

Proline concentration increased significantly and proportionally to the concentration of NaCl and mannitol in the medium. However, a highly significant difference was recorded between the effects of NaCl- and mannitol- induced stress (Table 1). Thus, mannitol-treated calli accumulated proline at less extent than the salt-treated ones. The accumulation differed significantly among the cvs., and we observed that the sensitive cv. accumulated more proline than the stress-resistant cv. under both NaCl- and mannitol-induced stress. Thus, at the highest NaCl concentration, proline accumulation increased by ~20, 17 and 13-fold in comparison of the control in R570, CP59-73 and NCo310, respectively. While it increased about 13-fold in CP59-73

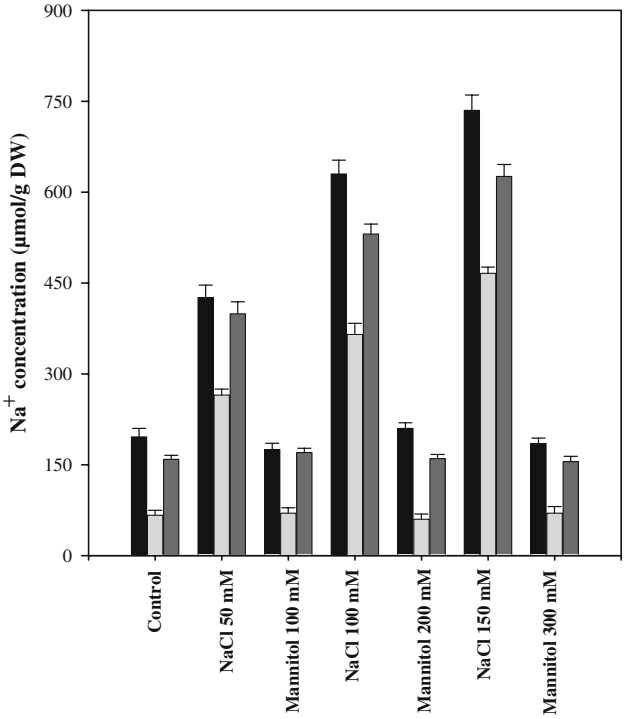

Fig. 3 Effect of NaCl and mannitol induced stress on Na^+ concentration in sugarcane (*Saccharum* sp.) cvs. NCo310, R570 and CP59-73 calli after 4 weeks of exposure to stress. Each value is the mean of six replicates and *vertical bars* represent ±standard error

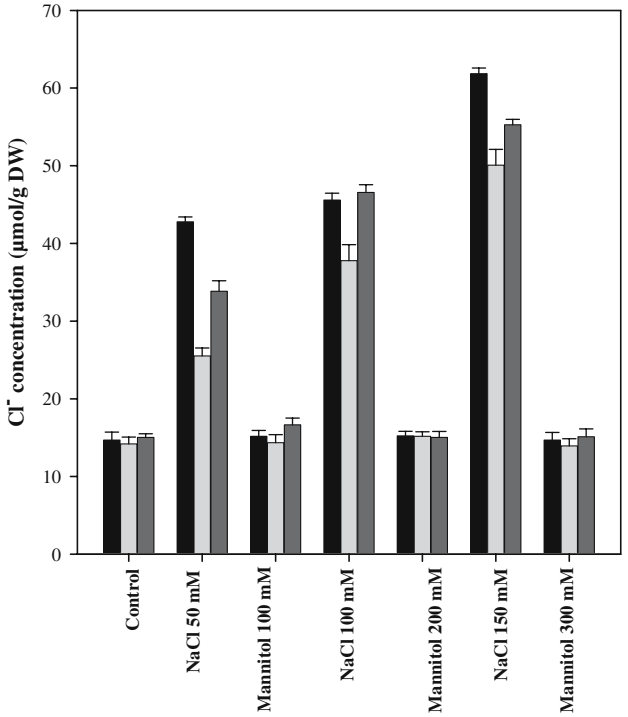

Fig. 4 Effect of NaCl and mannitol induced stress on Cl^- concentration of sugarcane (*Saccharum* sp.) cvs. NCo310, R570 and CP59-73 calli after 4 weeks of exposure to stress. Each value is the mean of six replicates and *vertical bars* represent ±standard error

calli, and tenfold in both NCo310 and R570 calli in comparison to the control under mannitol iso-osmotic stress (Fig. 7).

Discussion

The application of NaCl- and mannitol-induced stress decreased considerably either RGR and water content values among all cvs. However, a highly significant difference was recorded among the effects triggered by each kind of stress. The highest RGR and water content decrease were noticed under mannitol-induced osmotic stress, which indicated that RGR inhibition is due to the reduction of water availability and the loss of turgor in both CP59-73 and NCo310 cvs., as reported previously in other species (Lutts et al. 1996b, Mohamed et al. 2000; Watanabe et al. 2000). In contrast, R570 seemed to preserve a highest RGR value under mannitol-induced osmotic stress. These findings allow us to conclude that at the plant cell level, NCo310 is the most salt-resistant and R570 is the most salt-sensitive while CP59-73 displays an intermediary behaviour. Under mannitol-induced osmotic stress, NCo310 and R570 are considered as a drought-resistant cvs., while CP59-73 is relatively drought-sensitive cv. These results corroborate with those obtained at the whole plant level in relation to salt stress (unpublished data). While the data relating to the drought resistance of the cvs. at the whole plant level are scarce.

The fact that NaCl affected the RGR value at least extent is mostly due to the selective accumulation of Na^+ and Cl^- ions. A moderate increase of Na^+ and Cl^- within callus tissue might avoid water loss and ensure an economic way to adjust osmotically (Dutta Gupta et al. 1995; Chinnusamy and Zhu 2003; Benlloch-Gonzàlez et al. 2005). However, when the ability of the cells to compartmentalize the ions into the vacuole is exceeded, ions build up in the cytoplasm and lead to severe ion imbalances and to conformational changes in the plasma membrane electrical potential (Chinnusamy and Zhu 2003; Sairam and Tygai 2004). Calli of the salt-resistant NCo310 accumulated more Cl^- than calli obtained from the salt-sensitive R570 as was reported in tomato (Cano et al. 1996) and rice (Lutts et al. 1996b). Such a high accumulation in the salt-resistant cv. apparently did not cause much injury

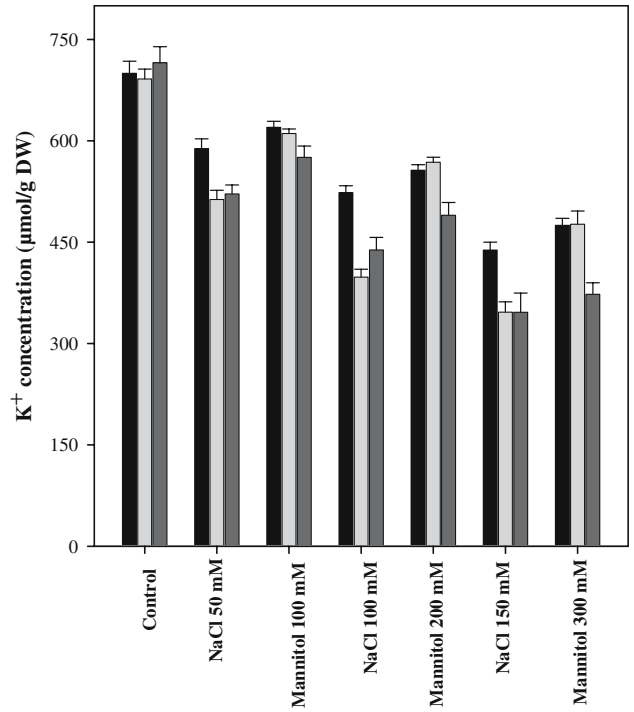

Fig. 5 Changes in K^+ concentration of sugarcane (*Saccharum* sp.) cvs. NCo310, R570 and CP59-73 calli as affected by NaCl and mannitol induced stress after 4 weeks of exposure to stress. Each value is the mean of six replicates and *vertical bars* represent ±standard error

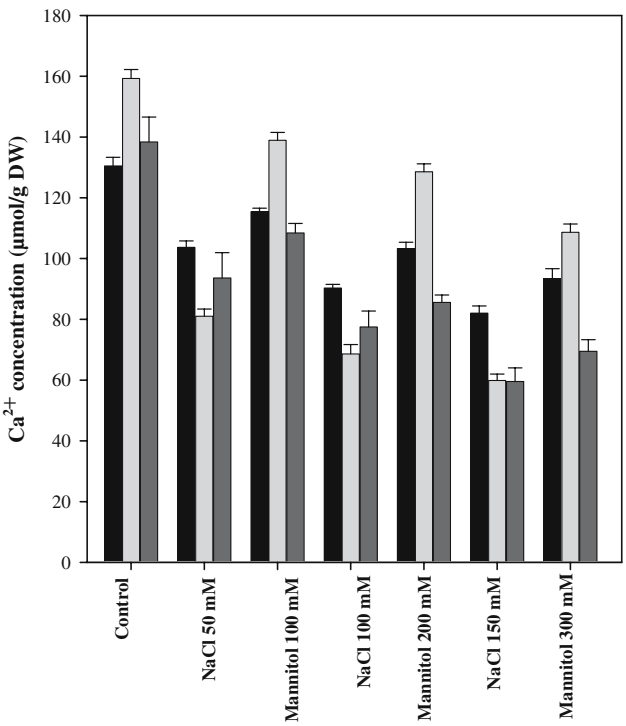

Fig. 6 Changes in Ca^{2+} concentration of sugarcane (*Saccharum* sp.) cvs. NCo310, R570 and CP59-73 calli as affected by NaCl and mannitol induced stress after 4 weeks of exposure to stress. Each value is the mean of six replicates and *vertical bars* represent ±standard error

to the calli as revealed by the RGR data. These statements allow us to conclude that salinity resistance in sugarcane cvs. is ascribed, at least partly, to the restriction of Na^+ accumulation and also to suggest that salt-resistant NCo310 has developed an exclusion mechanism to cope with the presence of salt in the medium (Greenway and Munns 1980). In contrast, the salt sensitivity of R570 cv. could be due to the lack of efficient compartmentation of Na^+ ions, which build up in the cytoplasm until reaching critical levels. However, the attribution of the deleterious effects of salt stress solely to the Na^+ toxicity might be an oversimplification of the events taking place during the exposure to salinity (Lutts et al. 1996b). Under salt-induced stress, the increase in Na^+ concentration and the subsequent decrease in K^+ concentration observed among sugarcane cvs. were previously reported in several other species (Basu et al. 2002; Benlloch-Gonzàlez et al. 2005). This could be due to the fact that some species were able to substitute K^+ by Na^+ to ensure the osmotic adjustment (Rus et al. 1999). Under mannitol-induced osmotic stress, the Na^+ and Cl^- concentration were not affected. In contrast, a substantial decrease of K^+ was recorded among the cvs., as was reported in wheat (Trivedi et al.1991). We also observed that the stress-resistant cvs. maintained a higher amount of K^+ than the stress-sensitive under both stresses. These findings corroborate with those reported in rice (Lutts et al. 1996b) and wheat (Lutts et al. 2004). We also observed that under both salt- and mannitol-induced stress, Ca^{2+} concentration decreased significantly but at least extent under mannitol-induced stress as was reported in other species (Lutts et al. 1996b, 2004). Among cvs., the reduction was more important in the stress-sensitive than in the stress-resistant cv. under both stresses. It is well known that Ca^{2+} plays a crucial role in the regulation of ion uptake and promotes the K^+ versus Na^+ uptake (Hirschi 2004). Thus, the highest uptake of Na^+ and leakage of K^+ observed in the salt-sensitive cv. could be related to the strong diminution of the Ca^{2+} concentration in the callus cells (Grattan and Grieve 1999). In summary, our results indicated clearly that the disruption of the ion concentration occurred under both salt- and mannitol- induced stress. Moreover, and from a quantitative point of view, the inorganic fraction did not contribute to the osmotic adjustment in the presence of mannitol-induced osmotic stress, while the Na^+ and Cl^- seemed to be the

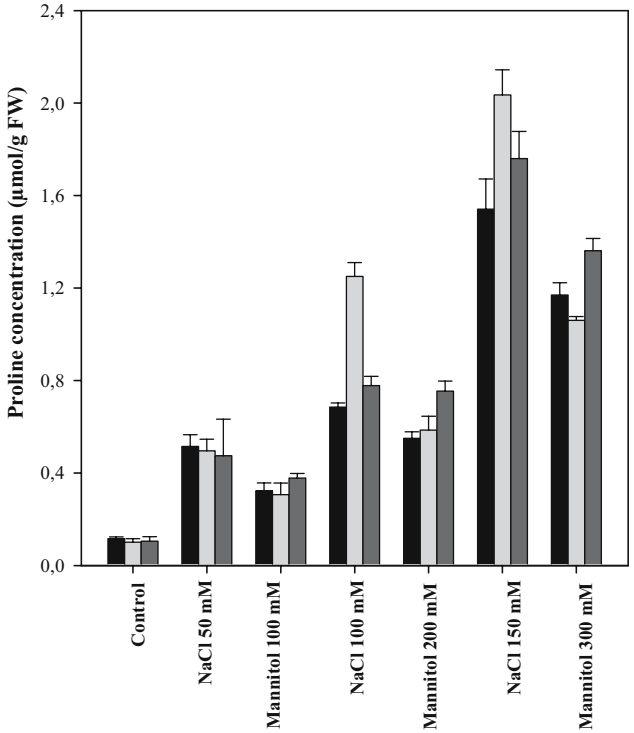

Fig. 7 Changes in proline concentration of sugarcane (*Saccharum* sp.) cvs. NCo310, R570 and CP59-73 calli as affected by NaCl and mannitol induced stress after 4 weeks of exposure to stress. Each value is the mean of six replicates and *vertical bars* represent ±standard error

major contributors to osmotic adjustment under salt induced stress.

Another strategy that plant adopt to withstand stress conditions is the accumulation of compatible solutes (Delauney and Verma 1993). These compounds, including proline, appeared to have a little or no interfering effects on macromolecules functioning (Hasegawa et al. 2000). In sugarcane cvs., proline concentration increased considerably under salt- and mannitol-induced stress, which indicates that the proline overproduction is a non-specific response (Bajji et al. 2000; Alvarez et al. 2003; Ashraf and Harris 2004). A significant difference was recorded among the effect of each kind of stress on proline accumulation, with the highest increase under NaCl-induced stress. Thus, the greatest accumulation of proline is thought to be due to the osmotic component of salt stress and that specific ions could influence the extent of this response (Lutts et al. 1996b, Benlloch-Gonzàlez et al. 2005). However, the precise role of proline accumulation in stress resistance remains a matter of debate and up to now, the published reports allow no evidence whether proline is the cause or the consequence of the metabolic adaptation to stress (Ashraf and Harris 2004). Our results revealed that under both stresses, the stress-resistant cv. accumulated proline at lesser extent than the stress-sensitive one. This finding let us suggest that proline accumulation among sugarcane cvs. is merely a symptom of injury rather than a stress resistance trait. Identical statements were reported in several other species (Cano et al. 1996; Garcia et al. 1997; Tonon et al. 2004). Furthermore, the contribution of proline to osmotic adjustment, from a quantitative point of view, in sugarcane cvs. seemed to be insignificant as was reported previously in other species under mannitol- and NaCl- induced stress (Mohamed et al. 2000; Benlloch-Gonzàlez et al. 2005). In contrast, the assumption that proline is a stress resistance marker has been widely adopted (Alvarez et al. 2003; Ehsanpour and Fatahian 2003). As well, proline can serve as an organic nitrogen reserve ready to be used after stress relief to sustain both amino acid and protein synthesis (Trotel et al. 1996; Sairam and Tygai 2004).

Conclusion

The present study highlights the importance of the effects of both ionic and osmotic component of the salt stress on sugarcane callus. Our results lead us to suggest that the physiological mechanisms that mediate the response to salt and drought stress are different. We also provide evidence that the growth inhibition is mainly due to the loss of turgor under mannitol-induced stress and to the build up of Na^+ and Cl^- ions in the cytoplasm under salt stress. Moreover, we demonstrated that the ion status is closely related to the nature of the stress factor applied in the medium. Among cvs., we revealed that stress resistance in sugarcane cvs. is closely related to the retention of an adequate water status, a high amount of K^+ and Ca^{2+} and a low level of Na^+. We also showed that proline is not directly involved in stress resistance at the cellular level in sugarcane.

Acknowledgments The authors are very grateful to D. El Ghrassli (Centre Technique des Cultures Sucrières, Morocco) for kindly providing the sugarcane cultivars. This research was financially supported by funds of Programme d'Appui à la Recherche Scientifique (PARS AGRO 180) from the Ministère de l'Enseignement Supérieur, de la Formation des Cadres et de la Recherche Scientifique of Morocco.

References

Alvarez I, Tomaro LM, Benavides MP (2003) Changes in polyamines, proline and ethylene in sunflower calluses treated with NaCl. Plant Cell Tissue Organ Cult 74:51–59

Ashraf M, Harris PJC (2004) Potential biochemical indicators of salinity tolerance in plants. Plant Sci 166:3–16

Bajji M, Lutts S, Kinet JM (2000) Physiological changes after exposure to and recovery from polyethylene glycol-induced water deficit in callus cultures issued from durum wheat (*Triticum durum* Desf.) cultivars differing in drought resistance. J Plant Physiol 156:75–83

Basu S, Gangopadhyay G, Mukherjee BB (2002) Salt tolerance in rice in vitro: implication of accumulation of Na^+, K^+ and proline. Plant Cell Tissue Organ Cult 69:55–64

Bates LS, Waldren RP, Teare ID (1973 Rapid determination of free proline for water-stress studies. Plant Soil 39:205–207

Benlloch-Gonzàlez M, Fournier JM, Ramos J, Benlloch M (2005) Strategies underlying salt tolerance in halophytes are present in *Cynara cardunculus*. Plant Sci 168:653–659

Cano EA, Perez-Alfocea F, Moreno V, Bolarin MC (1996) Responses to NaCl stress of cultivated and wild tomato species and their hydrids in callus cultures. Plant Cell Rep 15:791–794

Chinnusamy V, Zhu JK (2003) Plant salt tolerance. Top Curr Genet 4:241–270

Delauney AJ, Verma DPS (1993) Proline biosynthesis and osmoregulation in plants. Plant J 4:215–223

Dutta Gupta S, Auge RM, Denchev PD, Conger BV (1995) Growth, proline accumulation and water relations of NaCl-selected and non-selected callus lines of *Dactylis glomerata* L. Environ Exp Bot 35:83–92

Ehsanpour AA, Fatahian N (2003) Effects of salt and proline on *Medicago sativa* callus. Plant Cell Tissue Organ Cult 73:53–56

Flowers TJ, Yeo AR (1995) Breeding for salinity resistance in crop plants; what the next? Aust J Plant Physiol 22:875–884

Garcia AB, Engler JDA, Iyer S, Gerats T, Montagu MV, Caplan AB (1997) Effects of osmoprotectants upon NaCl stress in rice. Plant Physiol 115:159–169

Greenway H, Munns R (1980) Mechanisms of salt tolerance in nonhalophytes. Annu Rev Plant Physiol 31:149–190

Grattan SR, Grieve CM (1999) Salinity-mineral nutrient relations in horticultural crops. Sci Hortic 78:127–157

Guerrier G, Patolia JS (1989) Comparative salt responses of excised cotyledons and seedlings of pea to various osmotic and ionic stresses. J Plant Physiol 135:330–337

Hasegawa P, Bressan RA, Zhu JK, Bohnert HJ (2000) Plant cellular and molecular responses to high salinity. Annu Rev Plant Mol Biol 51:463–499

Hirschi KD (2004) The calcium conundrum. Both versatile nutrient and specific signal. Plant Physiol 136:2438–2442

Lerner HR (1985) Adaptation to salinity at the plant cell level. Plant Soil 89:3–14

Lutts S, Almansouri M, Kinet JM (2004) Salinity and water stress have contrasting effects on the relationship between growth and cell viability during and after stress exposure in durum wheat callus. Plant Sci 167:9–18

Lutts S, Kinet JM, Bouharmont J (1996a) Effects of salt stress on growth, mineral nutrition and proline accumulation in relation to osmotic adjustment in rice (*Oryza sativa* L.) cultivars differing in salinity resistance. Plant Growth Regul 19:207–218

Lutts S, Kinet JM, Bouharmont J (1996b) Effects of various salts and of mannitol on ion and proline accumulation in relation to osmotic adjustment in rice (*Oryza sativa* L.) callus cultures. J Plant Physiol 149:186–195

Mohamed MAH, Harris PJC, Henderson J (2000) In vitro selection and characterisation of a drought tolerant clone of *Tagetes minuta.* Plant Sci 159:213–222

Munns R, Husain S, Rivelli AR, Richard A, James RA, Condon AGT, Lindsay MP, Lagudah ES, Daniel P, Schachtman DP, Hare RA (2002) Avenues for increasing salt tolerance of crops, and the role of physiologically based selection traits. Plant Soil 247:93–105

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant 15:473–497

Paquin R, Lechasseur P (1979) Observations sur une méthode de dosage de la proline libre dans les extraits de plante. Can J Bot 57:1851–1854

Parida KA, Das AB (2005) Salt tolerance and salinity effects on plants. Ecotoxicol Environ Saf 60:324–349

Rus AM, Panoff M, Perez-Alfocea F, Bolarin MC (1999) NaCl responses in tomato calli and whole plants. J Plant Physiol 155:727–733

Sacchi AG, Morgutti S, Abruzzese A, Alisi C, Cocucci M, Espen L, Leva RA, Muleo R, Cocucci MS (1995) Changes in some physiological and biochemical parameters during two subcultures in Kiwi (*Actinidia deliciosa*) callus. Plant Sci 106:107–113

Sairam RK, Tygai A (2004) Physiology and molecular biology of salinity stress tolerance in plants. Curr Sci 86:407–421

SAS Institute Inc. (1988) SAS/STATJ User's Guide. Release 6.03 edn. SAS Institute Inc., Cary, NC, p.1028

Tonon G, Kevers C, Faivre-Rampant O, Graziani M, Gaspar T (2004) Effect of NaCl and mannitol iso-osmotic stresses on proline and free polyamine levels in embryogenic *Fraxinus angustifolia* callus. J Plant Physiol 161:701–708

Trivedi S, Galiba G, Sankhla N, Erdei L (1991) Responses to osmotic and NaCl stress of wheat varieties differing in drought and salt tolerance in callus cultures. Plant Sci 73:227–232

Trotel P, Bouchereau A, Niogret MF, Larher F (1996) The fate of osmoaccumulated proline in leaf discs of rape (*Brassica napus* L.) incubated in a medium of low osmolarity. Plant Sci 118:31–45

Wahid A, Rao AR, Rasul E (1997) Identification of salt tolerance traits in sugarcane lines. Field Crops Res 54:9–17

Watanabe S, Kojima K, Ide Y, Sasaki S (2000) Effects of saline and osmotic stress on proline and sugar accumulation in *Populus euphratica* in vitro. Plant Cell Tissue Organ Cult 63:199–206

Zhu JK (2001) Plant salt tolerance. Trends Plant Sci 6:66–71