Belg. Journ. Bot. **138** (2) : 173-180 (2005)

EFFECTS OF NaCl ON GROWTH AND ION AND PROLINE ACCUMULATION IN SUGARCANE (*SACCHARUM SP.*) CALLUS CULTURE

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Received 24 November 2004; accepted 1 April 2005.

ABSTRACT. — The effects of salt on growth, ion and proline accumulation were investigated *in vitro* in two sugarcane cultivars : NCo310 (salt-resistant) and CP65-357 (salt-sensitive). Leaf explant-derived calli obtained from the two sugarcane (*Saccharum* sp.) cultivars were exposed to four concentrations of NaCl (0, 34, 68 and 102 mM). Relative growth rate, ion concentrations and proline accumulation were quantified after 4 weeks of stress. NaCl inhibited growth in the two cultivars but to a lower extent in calli issued from NCo310 comparatively to calli obtained from CP65-357. In response to salinity, Na^+, Cl^- and proline concentrations increased significantly in calli of both cultivars while K^+ concentration decreased. The two cultivars accumulated similar quantities of Na^+; the highest accumulation of Cl^- occurred in calli of salt-tolerant NCo310 coupled with the lowest decrease in K^+ concentration. Calli issued from CP65-357 accumulated more proline than those of NCo310. These results suggested an implication of Cl^- and K^+ in salt resistance at cellular level in these genotypes and that proline is a symptom of injury in stressed sugarcane calli rather than an indicator of resistance.

KEY WORDS. — *Saccharum*, growth, ion concentration, proline, salt-resistance.

ABBREVIATIONS. — d.m. = dry matter; f.m. = fresh matter ; RFWG = mean relative growth rate.

INTRODUCTION

Salinity is a major environmental factor limiting crop productivity in the arid and semi-arid areas of the world (DASGAN *et al.* 2002). This complex abiotic stress, which presents an osmotic and an ionic component, induces a wide range of metabolic perturbations in higher plants, which in turn result in growth reduction and alteration of nutritional balance. To survive in the presence of salt, plants have developed several adaptative mechanisms whose comprehension remains incomplete (LUTTS *et al.* 1996a). There is substantial variation in salt-resistance among different species (MUNNS *et al.* 2002) but also among cultivars within a given species (WATANABE *et al.* 2000, AL-KARAKI 2000, GHOULAM *et al.* 2002). Generally, the presence of NaCl in plant environment or in culture medium induce an increase in Na^+ and Cl^- concentrations and a decrease in K^+ concen-

tration in shoots and roots. In many glycophyte species, the salt-resistant genotypes accumulate less toxic ions (Na^+ and/ or Cl^-) in growing tissue than the salt-sensitive ones (LUTTS *et al.* 1996a,b, ALMANSOURI *et al.* 1999, ASHRAF & AHMAD 2000). In some plants, however, resistance may be linked to a physiological strategy of salt-tolerance involving the inclusion of toxic ions. *Vigna radiata* (GULATI & JAIWAL 1993) and *Lens culinaris* (ASHRAF & WAHEED 1993) belong to this category. Salt-tolerance is usually associated with the compartmentation of toxic ions in vacuole in order to keep in the cytoplasm only low concentrations of Na^+ compatible with enzymatic activities. Another frequently reported plant response to salinity consists in a decrease in K^+ concentration. Potassium is the most abundant cation in higher plants and an important macroelement for cellular metabolism (MÄSER *et al.* 2002). Generally, the most salt-resistant genotypes maintain a high supply of K^+ in the presence of an excess of Na^+ (LUTTS & GUERRIER 1995, SANTA-MARIA & EPSTEIN 2001).

Proline accumulation is frequently reported in plants exposed to salt or water stress, but the role of accumulation still remains unclear (LUTTS *et al.* 1996a). Cytoplasmic accumulation of this amino acid may be involved in the osmotic adjustment between cytoplasm and vacuole (DELAUNEY & VERMA 1993, KAVI KISHOR *et al.* 1995). Proline is also supposed to act as a protective agent of enzymes (SOLOMON *et al.* 1994) and intracellular structures (VAN RENSBURG *et al.* 1993) or as a free radical scavenger (ALIA SARRADHI & MOHANTY 1993). Proline accumulation may also be an attempt to regulate cytosolic pH (VENEKAMP 1989). However, in some experimental systems proline could be a symptom of the stress (LUTTS *et al.* 1996a). For sugarcane, in contrast to other glycophytes such as rice, corn or wheat, little is known about the physiological mechanisms implicated in salt resistance. For this plant considered as moderately sensitive to salinity (MAAS & HOFFMANN 1990), some studies analysed salt effects on whole plant growth and metabolism (MEINZER *et al.* 1994, ROZEFF 1995, LINGLE *et al.* 2000), but until now mineral nutrition and osmotic adjustment have rarely been considered at the cell level.

In vitro tissue culture constitutes an important tool to study the physiological mechanisms of salt resistance at the cellular level because it allows a control of the homogeneity of the stress. In the present study, we compared NaCl effects on growth, as well as on Na^+, Cl^-, K^+, and proline concentrations of calli obtained from two sugarcane cultivars that differ in their response to salt stress at cellular level. It aims to analyse the implication of ions and proline accumulation in sugarcane salt tolerance *in vitro*.

MATERIAL & METHODS

PLANT MATERIAL AND CULTURE CONDITIONS

The two sugarcane (*Saccharum* sp.) cultivars were obtained from the Technical Center of Sugar's Cultures (CTCS), Morocco. CP65-357 (Canal Point) is an American cultivar largely cultivated in Morocco and with a salt-sensitive behaviour compared to the South African cultivar Natal Coimbatore (NCo310) (GANDONOU *et al.*, unpubl.). Stalk segments were surface-sterilised with ethanol 70% and sown in pots containing sand, and grown in a greenhouse with a natural illumination (150 $\mu mol\ m^{-2}\ s^{-1}$). The day/night temperature was about 27°C/22°C. Daytime humidity was between 70 and 80%. Irrigation was performed every two days. Sugarcane plants were grown in these conditions for approximately 6 months.

The explants used for callus induction were leaf segments issued from the sheath of the youngest leaves. The basal part of the stem was surface-sterilised for 10 min in 0.03% mercuric chloride added with Tween 80, followed by three rinses in sterile distilled water (10 min each). After drying on sterile filter paper, leaf segments were aseptically placed on MS medium (MURASHIGE & SKOOG 1962) supplemented with 3 mg L^{-1} 2,4- dichlorophenoxyacetic acid and 30 g L^{-1} sucrose. The pH was ajusted to 5.8 with NaOH (0.1 N) and all media were solidified with 8 g L^{-1} agar before autoclaving during 20 min at 120 °C. Five explants were cultivated per jar and the cultures were kept in darkness at 25 ± 1°C.

IN VITRO SALT TREATMENT AND GROWTH DETERMINATION

After two subcultures (4 weeks each), the growing calli were transferred to callus culture media containing 0, 34, 68 or 102 mM NaCl. Calli were maintained on these media for 4 weeks in the same environmental conditions as described above. Calli were weighted

before being transferred to these media (W_0) and weighted again after the 4 weeks of treatment (W_1). Relative growth rates of calli (RFWG) were calculated as $(W_1 - W_0)/ W_0$.

Extraction and quantification of ion concentrations

Calli were rinsed for 5 min in cold distilled water in order to remove free ions from the apoplasm without substantial elimination of cytosolic solutes as recommanded by Sacchi *et al.* (1995). Calli were then oven-dried at 80°C for 48 h and ground in a mortar. The resulting powder was dried again for 24 h. For Na^+ and K^+ quantification, calli were digested in HNO_3 and analysed using a flame spectrophotometer (PHF 90 D). For Cl^- content estimation, ions were extracted with hot distilled water (80°C during 2 h). Chloride was determined colorimetrically with ferric ammonium sulfate and mercuric thiocyanate according to Guerrier & Patolia (1989). Ion concentrations are expressed as percentages of dry matter.

Extraction and quantification of free proline

For proline quantification, 200 mg of tissue per callus were ground in a mortar, homogenised in 4 ml of methanol-chloroform-water (67%-28%-5%) at 0°C and centrifuged at 20,000 g for 30 min. The supernatants were then incubated at 4 °C for 12 h in the presence of 0.25 mL chloroform and 0.9 mL distilled water. Proline was quantified spectrophotometrically (515 nm) in the upper phase using ninhydrin acid (Bates *et al.* 1973) and expressed as percentages of fresh matter. L-proline (Sigma Chemical) was used as standard.

Statistical analyses

All the measurements (growth, ions and proline concentrations) were repeated on two sets of 30 to 35 calli (5 per jar) with similar results. Data presented are those of one the two experiments. Each value is presented in the form of mean ± standard error with a reading of four (growth) or three (ions and proline concentrations) independent samples per treatment. The analysis of the main effects of cultivars and stress intensity was based on a 2-way analysis of variance (ANOVA). For each cultivar, the pairwise correlations between the various parameters (RFWG, Na^+, K^+, Cl^- and proline) were tested using Spearman rank correlation coefficients (r). All statistical analyses were performed using SAS 92.

RESULTS

Growth

In the absence of stress, the mean relative growth rate (RFWG) of calli obtained from NCo310 was significantly higher than that of calli originating from CP65-357 (Fig.1). NaCl application induced a significant decrease of callus growth in both cultivars. Difference between the two cultivars was highly significant (Table 1). For CP65-357, callus RFWG declined from 1.192 in the control to about 0.734, 0.66 and 0.56 at 34, 68 and 102 mM NaCl, respectively, which corre-

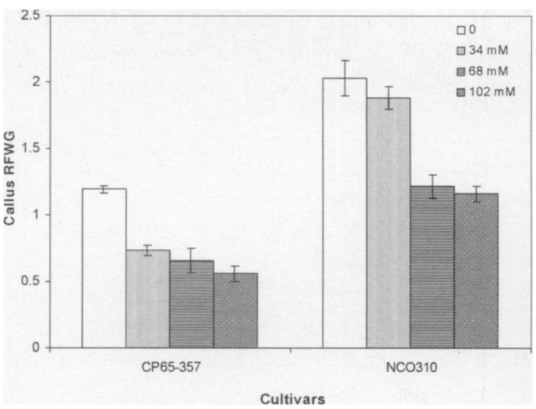

Fig.1. — Relative growth rate (RFWG ; in %) of calli obtained from two sugarcane cultivars (CP65-357, salt-sensitive and NCo310, salt-resistant) and exposed during 1 month to different concentrations of NaCl. Vertical bars represent S.E.

Table 1

Two-way analysis of variance for RFWG, ion concentration and proline accumulation of sugarcane calli ; F- ratios are given for the main effects of the following levels of classification : stress intensity (i.e. NaCl concentration of stressing medium), cultivars and interaction between these levels of classification

	Stress intensity	Cultivar	Interaction
RFWG	28.27***	144.73***	4.32*
Na^+	277.07***	2.07^{ns}	0.37^{ns}
K^+	79.76***	37.85***	11.41***
Cl^-	303.9***	325.44***	45.80***
Proline	142.24***	43.12***	10.68***

Note. ^{ns} : not-significant ; * : $P < 0.05$; *** : $P < 0.001$.

sponds to 62%, 55% and 47% of the control, respectively. In the case of NCo310, callus RFWG declined from 2.032 in the control to 1.883, 1.215 and 1.163 at 34, 68 and 102 mM NaCl, respectively, which corresponds to 92%, 60% and 57% of the control, respectively. NCo310 thus appeared to be more salt-tolerant than CP65-357 at the cellular level.

Ion concentration

In the absence of stress, callus obtained from the two cultivars did not differ in Na^+ and K^+ concentrations (Fig. 2-A and B). As expected, a significant increase in Na^+ concentration was recorded in the presence of NaCl (Fig. 2-A) and the endogenous accumulation of this element was proportional to the NaCl concentration in the medium. Na^+ accumulation was similar for the two cultivars, whatever the NaCl concentration in the medium (Table 1). K^+ concentration decreased significantly under salt stress (Fig. 2-B) but there were highly significant differences between the

Table 2

K^+/Na^+ ratio of sugarcane calli obtained from two cultivars (CP65-357, salt-sensitive and NCo310, salt-tolerant) as affected by different NaCl concentrations

NaCl concentrations (mM)	Cultivars CP65-357	NCo310
0	12.72	11.79
34	1.75	2.67
68	0.83	1.24
102	0.29	0.70

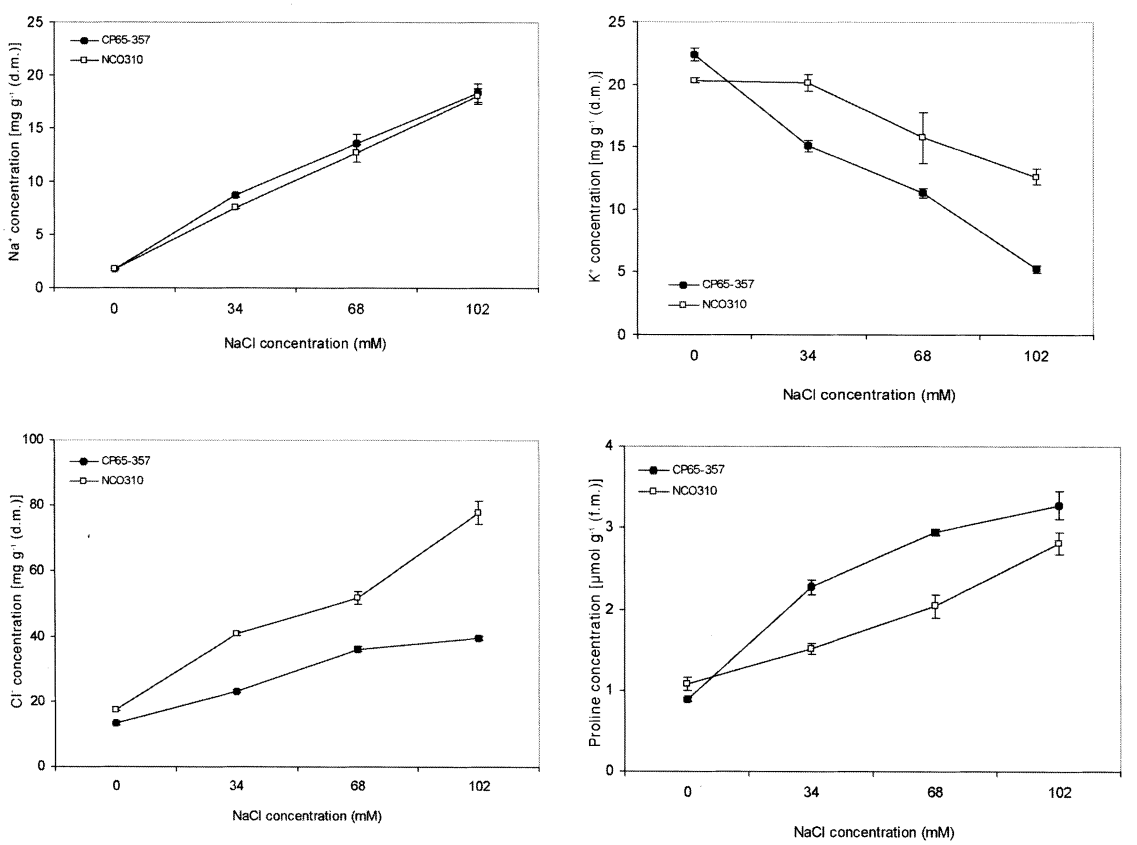

Fig. 2. — Sodium (A), potassium(B), chloride(C) and proline(D) concentrations of sugarcane calli obtained from two cultivars (CP65-357, salt-sensitive and NCo310, salt-resistant) and exposed during 1 month todifferent concentrations of NaCl. Vertical bars represent S.E.

two genotypes (Table 1). Indeed, K^+ concentrations for CP65-357 were about 67%, 50% and 23% of the control at 34, 68 and 102 mM NaCl, respectively, while they were about 99%, 77% and 62% of control at the same NaCl concentrations in the case of NCo310. Calli obtained from NCo310 thus maintained a higher internal K^+ concentration in the presence of NaCl compared to CP65-357. A very highly significant interaction between cultivar and stress intensity was also observed for this parameter (Table 1). The increasing of Na^+ amounts in calli issued from the two sugarcane genotypes coupled with decreasing in K^+ concentration resulted in a strong decrease in K^+/Na^+ ratios in presence of NaCl (Table 2). Such a decrease was more accentuated in calli of salt-sensitive CP65-357 than in those of salt-tolerant NCo310.

Chloride concentration in control calli was higher in NCo310 than in CP65-357 (Fig. 2-C). In response to NaCl, Cl^- concentration increased significantly in both genotypes but the difference among them, as well as the interaction between cultivars and stress intensity were always significant (Table 1). NCo310 accumulated more Cl^- than CP65-357 under salt conditions. In calli obtained from CP65-357, Cl^- concentration increased by about 72%, 170% and 183% at 34, 68 and 102 mM NaCl, respectively, while in calli from NCo310, the Cl^- increase culminated at 133%, 196% and 344% for the same NaCl concentrations. (Fig. 2-C).

Free proline accumulation

No significant difference in proline concentration was recorded in the absence of stress (Fig. 2-D). In the presence of NaCl, free proline concentration increased significantly in calli obtained from both sugarcane cultivars. A very highly significant difference was recorded between cultivars (Table 1), with calli of the salt-sensitive cultivar CP65-357 accumulating more proline than those obtained from NCo310.

Analysis for multiple correlation

Multiple correlation study (Table 3) revealed a high correlation for all pairwise combinations of parameters ($| r | > 0.88$). For both genotypes exposed to NaCl, a significant positive correlation was observed between Na^+ and Cl^- on the one hand, and proline accumulation on the other hand. Growth was negatively correlated to proline concentration in the salt-sensitive cultivar CP65-357.

DISCUSSION

Salt-induced RFWG decrease was larger and detectable at lower doses in CP65-357 than in NCo310. These results are in agreement with our previous results (Gandonou *et al.* unpublished results), which indicated that NCo310 is more salt-resistant than CP65-357. Our results demonstrate that the salt resistance of this genotype may be, at least partly, due to a cellular component. In other sugarcane genotypes, Gonzalez *et al.* (1995) have shown using *in vitro* cell suspensions that NaCl stress reduces cell survival rate in all genotypes tested and found significant differences among them.

The effect of salt stress on plants and calli depends on three interacting components : i)

Table 3

Pairwise correlation between RFWG, Na^+, K^+, Cl^- and proline concentrations after salt treatment of calli obtained from two sugarcane cultivars (CP65-357, salt-sensitive and NCo310, salt tolerant)

	CP65-357					NCo310			
	Proline	Cl^-	K^+	Na^+		Proline	Cl^-	K^+	Na^+
RFWG	-0.985*	-0.919^{ns}	0.942^{ns}	-0.944^{ns}	RFWG	-0.917^{ns}	-0.885^{ns}	0.948^{ns}	-0.937^{ns}
Na^+	0.979*	0.983*	-0.996**		Na^+	0.989*	0.991**	-0.946^{ns}	
K^+	-0.970*	-0.964*			K^+	-0.970*	-0.925^{ns}		
Cl^-	0.973*				Cl^-	0.989*			

Note. ^{ns} : not-significant ; * : $P < 0.05$; ** : $P < 0.01$.

dehydration of the cells in response to the low external water potential, ii) nutritional imbalance caused by the interference of saline ions with essential nutrients and iii) toxicity due to the high accumulation of Na^+ and Cl^- in the cytoplasm.

Calli issued from the two *Saccharum* cultivars accumulated high amounts of Na^+ and Cl^- when exposed to NaCl and these ions are known to be toxic to cell metabolism. Our results revealed no difference between cultivars in Na^+ accumulation under salt-stress despite a highly significant difference observed between their growth rates. This result suggests that the detrimental effect of Na^+ on growth was higher in calli obtained from the sensitive CP65-357 than in those issued from NCo310. It was not possible in the present study to determine if calli obtained from the resistant cultivar NCo310 sequestrated Na^+ more efficiently in the vacuoles. Calli of the salt-tolerant NCo310 also accumulated more Cl^- than calli obtained from the sensitive CP65-357. Such a high accumulation in the resistant cultivar apparently did not cause much injury to the calli as reflected from the data of tissue growth. Chloride is generally disregarded in the studies related to salt resistance mechanisms. In our study, calli obtained from CP65-357 seemed to be particularly sensitive to putative Cl^- toxicity since salt-resistant NCo310 calli accumulate more Cl^-. Specific ion toxicity thus appears as an important part of NaCl effect on callus growth. It may be concluded that the internal concentration of accumulated ions is not the only factor to consider but that the metabolic consequence of such an accumulation may vary among cultivars of a given species. These data also suggest that the salt resistance of NCo310 is partly due to physiological tolerance rather than to an efficient selectivity in terms of ion absorption. For a given species, however, strategies of physiological resistance to salinity may differ according to the genotypes. In rice, Lutts *et al.* (1996a) reported that calli obtained from a salt-resistant cultivar accumulated less Na^+ and Cl^- than those produced from a sensitive cultivar and concluded to an exclusion mechanism. In other cultivars of rice, however, Basu *et al.* (2002) observed the opposite for Na^+ : calli of the salt-resistant SR-26B cultivar accumulated more Na^+ than calli of the sensitive Basmati 370.

In the presence of NaCl, there was a very highly significant difference between cultivars for K^+ concentration (Table 1). Calli of the salt-resistant NCo310 maintained a very high amount of this ion compared to those of the sensitive CP65-357. These results corroborate those reported in rice (Lutts *et al.* 1996a). Potassium ions are known to be a major component of osmotic adjustment during stress (Wu *et al.* 1996). Proline accumulation has also frequently been reported in salt-stressed calli and whole plants. Most usually, it has been considered to act as a compatible osmoticum and, therefore, to be involved in salt resistance mechanisms through its contribution to osmotic adjustment (Delauney & Verma 1993, Alvarez *et al.* 2003, Ehsanpour & Fatahian 2003). Other functions have been suggested for proline accumulation in stressed tissue : it could be a protective agent of enzyme and membranes (Solomon *et al.* 1994, Van Rensburg *et al.* 1993), a free radical scavenger (Smirnoff & Cumbes 1989), a storage compound for carbon and nitrogen (Jäger & Meyer 1977) or it could be involved in the regulation of cytosolic pH (Venekamp 1989). Our results suggest that it was not the case in the sugarcane calli we studied, since proline accumulation was higher and occurred at lower stress intensities in the salt-sensitive variety CP65-357 than in the salt-tolerant cultivar NCo310. Proline overproduction might then be a detrimental response to the osmotic component of salt stress and not an adaptative process as reported in rice (Lutts *et al.* 1996a), *Lycopersicon* species (Perez-Alfocea *et al.* 1994, Ruz-Alvarez & Guerrier 1994), maize (Ibarra-Caballero *et al.* 1988), soybean (Moftah & Michel 1987) and sorghum (Bhaskaran *et al.* 1985).

The multiple correlation analysis showed that accumulation of inorganic (Na^+, K^+ and Cl^-) and/or organic (proline) solutes in tissues were correlated. The fact that the correlation between Na^+ and K^+ on the one hand, and between Cl^- and K^+ on the other hand were negative and significant only in the case of the sensitive CP65-357 demonstrates that the detrimental effect of Na^+ and Cl^- on metabolism is mediated, at least partially, by K^+

loss. However, the correlation between growth and proline was negative and it was significant only in the case of salt-sensitive CP65-357 proving that proline accumulation did not contribute to salinity resistance.

The present study revealed that salt-resistance *in vitro* is genotype-dependent. For the first time in sugarcane, we have demonstrated that Cl^- toxicity is implied in salt effect at the callus level and that K^+ plays an important part in salt resistance *in vitro*. This study also showed that proline is not directly involved in salt resistance at the tissue level. Additional investigations are required to assess the physiological and biochemical basis of ion effects on stressed cell metabolism and the precise nature of salt tolerance mechanisms.

ACKNOWLEDGEMENTS

The authors thank Professor Mohamed ANALLA (FST, Tétouan) for his assistance in statistical analyses, Mr. Mohamed El GHRASSLI (CTCS, Morocco) for providing us with plant material. This research was 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.

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