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Callus growth and ion composition in response to long-term NaCl-induced stress in two sugarcane (*Saccharum* sp.) cultivars

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In this work, the effect of different concentrations of NaCl on calli induced from two sugarcane cultivars NCo310 and CP59-73 was studied. Growth and ion concentrations (Na^+, Cl^-, K^+ and Ca^{2+}) of calli were determined after 1, 2 and 3 months of stress with the objective to understand the cellular mechanisms operating in salt stress tolerance and to determine the implication of inorganic fraction in salt tolerance in sugarcane cultivars. A negative effect of the NaCl concentration and the duration of stress exposure on the callus rate growth was observed in both cultivars and with more extent in CP59-73 cv. Results showed an increase in Na^+ and Cl^- and a decrease in K^+ and Ca^{2+} concentrations after 1, 2 and 3 months of salt stress exposure. It also showed that resistant cv. NCo310 stressed calli accumulated less Na^+ and retained more K^+ and Ca^{2+} than CP59-73 calli. Cl^- appeared to be involved in osmotic adjustment since the resistant cv. NCo310 stressed calli accumulated more Cl^- than CP59-73 ones. These results suggested that the resistance to salinity in sugarcane is associated with a high K^+, Ca^{2+} and Cl^- concentrations and a low Na^+ concentration within cells.

Key words: sugarcane *(Saccharum* sp.), salt stress, ion uptake, callus growth, long-term stress exposure.

INTRODUCTION

Salinity is a significant factor that affects crop production and agricultural sustainability worldwide, since about 10% of the land surface and 50% of all irrigated land in the world are prone to salinity (Flowers et al., 2010). Salt stress affects several aspects of plant physiology by its osmotic and ionic components (Munns and Tester, 2008).

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However, the operating mechanisms remains till now poorly understood and it not easy to differentiate between the effects due to the osmotic component and those due to the ionic one (Gandonou et al., 2011). Adaptation to salt stress involves several mechanisms that help plants to adjust osmotically, to maintain a low cytoplasmic concentration of toxic ions and a high concentration of essential minerals (Munns, 2005).

It is well known that cell culture techniques, including callus culture constitutes an important tool to investigate the response to salts, such as NaCl, of several plants at cellular level (Perez-Alfocea et al., 1994; Lutts et al., 1996; Ehsanpour and Fatahian, 2003; Gandonou et al., 2011) and therefore, to understand the cellular mechanisms involved in the salt tolerance and/or sensitivity (Tal, 1983; Gandonou et al., 2011). As for sugarcane, there were a few studies on the implication of inorganic solutes to osmotic adjustment and salt tolerance at the cell level. In a previous study, it was shown that ions toxicity was implied in salt effect at cellular level and that K^+ ion plays a crucial role in sugarcane salt-tolerance (Gandonou et al., 2005a). In the present investigation, the effects of different NaCl concentrations (0, 50, 100 and 150 mM) and the duration of stress exposure on callus growth and ion concentration in two sugarcane genotypes, CP59-73 and NCo310, at the cellular level were studied.

MATERIALS AND METHODS

Plant material and culture conditions

Stalk segments of two sugarcane (*Saccharum* sp.) cultivars CP59-73 and NCo310 were surface sterilized with ethanol 70% for 5 min and were sown in plastic pots (20 cm/15 cm/10 cm) containing approximately 5 kg of soil under greenhouse conditions till reaching approximately 6 to 7 months. NCo310 is a salt-resistant cultivar and it is originated from South-Africa. While CP59-73 is originated from USA and it is largely cultivated in Morocco.

Calli were initiated from the youngest leaf segments as described by Gandonou et al. (2005a). The leaf segments were wiped with 70% (v/v) ethanol and sterilized with mercuric chloride $HgCl_2$ 0.03% (w/v) for 30 min and then rinsed 3 times with sterile distilled water. Explants were cultivated in Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 2 mg/l 2,4 Dichlorophenoxyacetic acid, 30 g/l sucrose and 8 g l^{-1} agar, before autoclaving during 20 min at 120°C. Five explants were cultivated per Petri dish (10 cm diameter/ 25 ml of medium per Petri dish). Cultures were kept in darkness at 25±1°C in a growth chamber. After 6 weeks of culture, calli were separated from the explant, individually weighted (from 100-200 mg) and then subcultivated in Petri dishes (5 calli per petri dish) on MS medium containing different concentrations of NaCl (0, 50, 100 and 150 mM). Subcultures were made every month during three months for further proliferation in the absence or in the presence of NaCl under culture conditions described previously. After each month of stress, both control and stressed calli were harvested for growth and mineral analysis. 20 to 25 calli were used for each treatment (cv. ·stress factor · duration of stress exposure).

Growth determination

After initiation, calli were separated from the explants and were weighed and then inoculated onto MS medium supplemented with different concentrations of NaCl. After each month of the salt stress exposure, control and stressed calli were weighed for relative growth rate (RGR) determination. Callus RGR was determined according to the formula; RGR = *[(final FW – initial FW)/initial FW]*.

Determination of ion concentration

The ions concentrations were determined using dry matter. Calli were rinsed for 5 min with cool distilled water to remove free ions from the apoplasm as recommended by Sacchi et al. (1995). Calli were then oven-dried at 80°C for 72 h and were grounded with a mortar. The ions Na^+, K^+ and Ca^{2+} were extracted after digestion with HNO_3 acid according to Lutts et al. (1996) and the extract was filtered. Na^+ and K^+ concentrations were determined as described by Gandonou et al. (2005a) using a flame spectrophotometer (Model PHF 90D, France). Ca^{2+} concentration was determined by atomic absorption spectrophotometer (Model AA-6200, Shimadzu, Kyoto, Japan) as described by Errabii et al. (2007). Chloride was extracted with hot distilled water (80°C for 2 h) as described by Gandonou et al. (2005a) and was determined spectrophotometrically at 470 nm as described by Guerrier and Patolia (1989) using ferric ammonium sulphate and mercuric thiocyanate.

Statistical analysis

The experiment was laid out as a Randomized Complete Design (RCD) with three factors and five replications. The three considered factors were cultivars (with two levels), NaCl concentrations (with four levels) and the duration of stress (with three levels). The experiments were repeated twice and gave similar trends. Each value is presented in the form of mean ± standard error with a reading of five independent samples per treatment. The analysis of the main effects of stress intensity, cultivars and the duration of stress was based on a three-ways analysis of variance (ANOVA). All statistical analyses were performed using SAS program (SAS Institute, 1992).

RESULTS

Callus relative growth rate

In absence of stress, the callus relative growth rate (RGR) is strongly influenced by genotype and we observed a significant difference between cultivars as shown in Table 1. NCo310 had higher RGR than CP59-73. However a decrease in RGR after the second and the third month of subculture was observed even in control calli (Figure 1).

In the presence of NaCl, The RGR decreased very significantly ($P < 0.001$) (Table 1) in both cvs with the increase of NaCl concentrations and the duration of the stress exposure but at lesser extent in cv. NCo310 calli than in CP59-73 ones. In the presence of 150 mM of NaCl, RGR reduction by 51, 60 and 65% in CP59-73 stressed calli and a reduction by 49, 49.5 and 58% in

Table 1. Three-ways analysis of variance (ANOVA) for RGR, Na^+, K^+ and Ca^{2+}) concentrations (µmol/g DW) and Cl^- concentration (mg/g DW) of sugarcane calli.

Parameter	RGR	Na^+	Cl^-	K^+	Ca^{2+}
Cv.	104.61***	1.77*	1.06^{ns}	25.76***	0.86^{ns}
Duration of stress exposure	91.43***	73.17***	33.70***	29.12***	4.68***
Concentration of NaCl	120.01***	96.24***	101.6***	78.77***	19.9***
Cv. X concentration of NaCl	27.12***	0.53^{ns}	5.38***	8.69***	2.14**
Cv. X duration of stress exposure	15.47***	8.74***	54.55***	1.17^{ns}	1.58*
Concentration of NaCl X Duration of stress	17.08***	57.84***	25.13***	19.30***	0.049^{ns}
Cv. X Concentration of NaCl X Duration of stress exposure	6.31***	2.84**	42.57***	1.59*	0.002^{ns}

F-values are given for the main effects of the following levels of classification: Cv. (cultivars), the duration of salt stress exposure, NaCl concentration and the interactions between these factors; ^{ns} not significant; *significant at p<0.05; ** significant at p<0.01; *** significant at p<0.001.

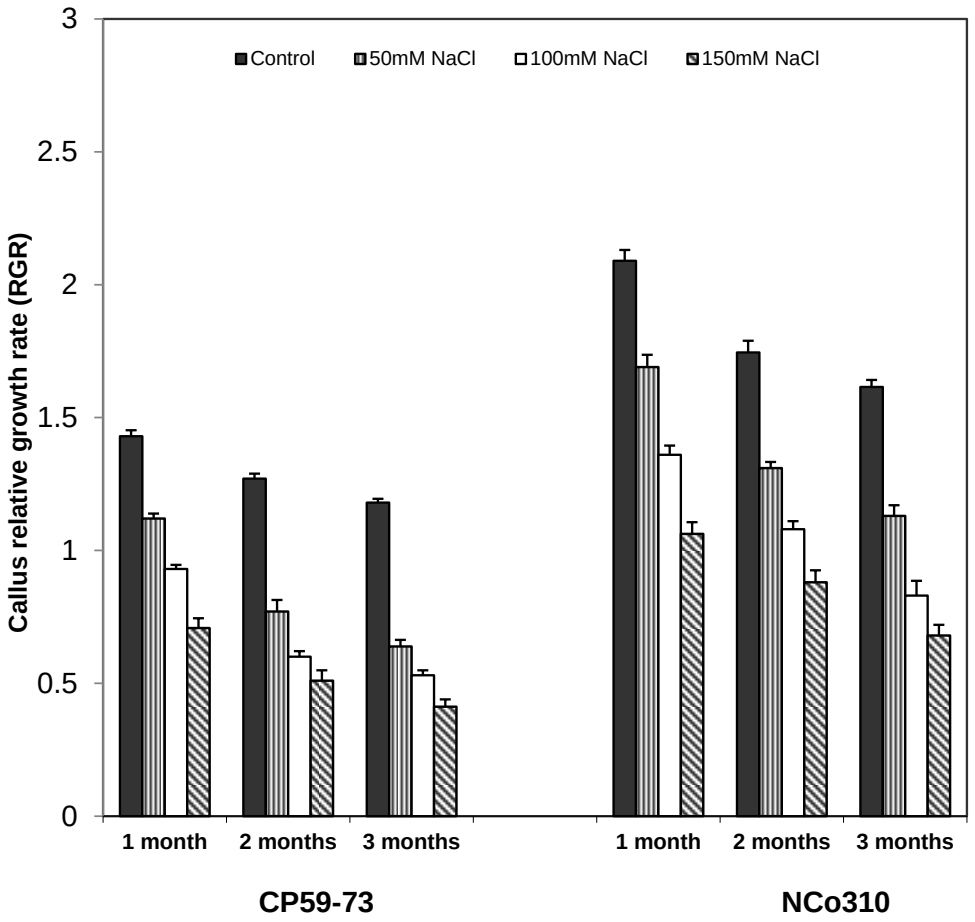

Figure 1. Changes in growth rate of sugarcane (*Saccharum* sp.) cvs CP59-73 and NCo310 calli as affected by NaCl induced stress after 1, 2 and 3 months of stress exposure. Vertical bars are means ±SEs, n=5.

NCo310 stressed calli, compared with the control was observed after 1, 2 and 3 months of salt-stress exposure, respectively (Figure 1).

Mineral analysis

Under saline conditions, both NaCl concentration and

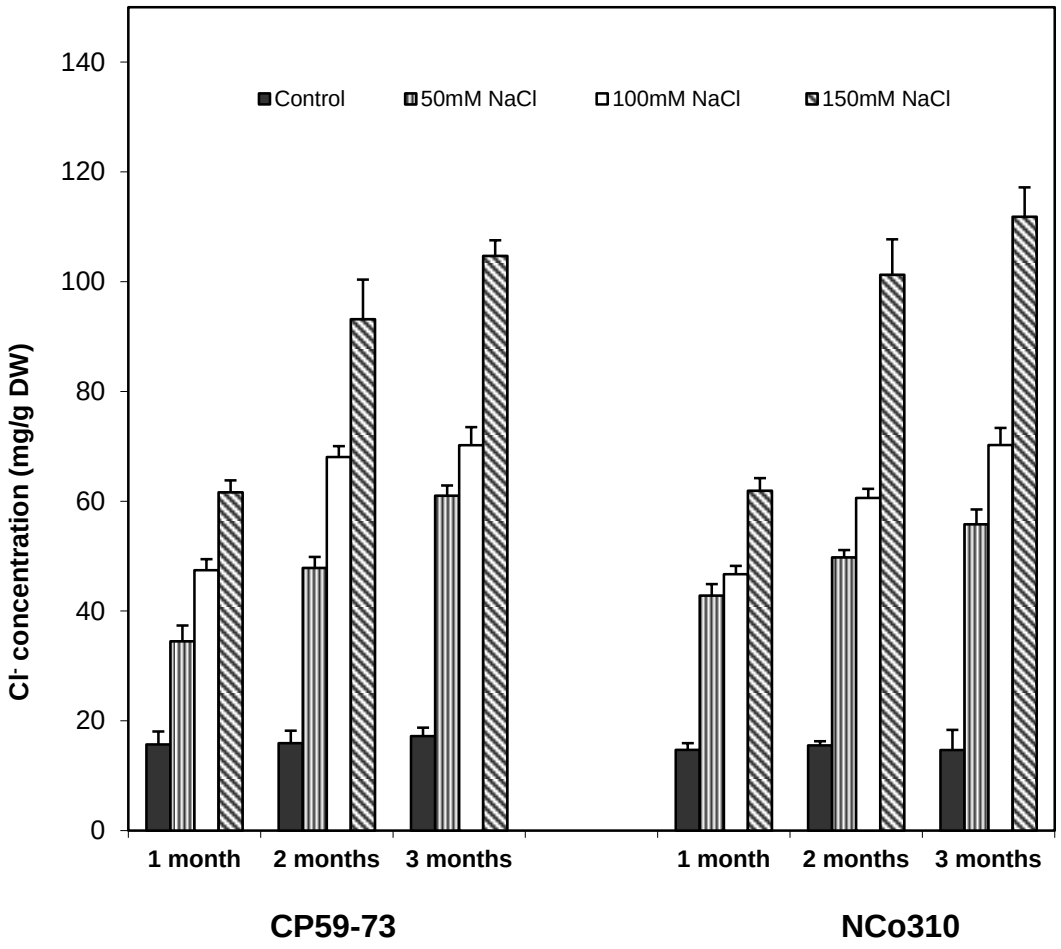

Figure 2. Effect of NaCl induced stress on Cl^- concentration in sugarcane (*Saccharum sp*.) cvs CP59-73 and NCo310 calli after 1, 2 and 3 months of stress exposure. Vertical bars are means ±SEs, n=5.

stress exposure duration exhibited a very significant effect on Na^+, Cl^-, K^+ and Ca^{2+} concentrations in sugarcane calli (Table 1).

The interaction between the effect of stress and cvs revealed that this interaction was non-significant for Na^+ concentration, and very significant for Cl^-, K^+ and Ca^{2+} (Table 1).

It was observed that Na^+ and Cl^- contents increased with the increase of NaCl concentrations and the duration of stress exposure. Thus, at the concentration of 150 mM, Cl^- concentration increased by about 293, 485 and 508% of the control in CP59-73 calli and to about 321, 552 and 661% compared to the control in NCo310 ones, respectively after 1, 2 and 3 months of stress exposure (Figure 2). It should be noted that the salt resistant NCo310 accumulated more Cl^- than CP59-73. In contrast, the accumulation of Na^+ was greater in CP59-73 stressed calli than in resistant cv. NCo310 ones. In fact, At the highest concentration of NaCl, the Na^+ concentration increased by about 294, 661 and 975.5% of the control in CP59-73 calli and by about 275, 503 and 804% of the control in NCo310 ones, respectively after 1, 2 and 3 months of salt-stress exposure (Figure 3). The increase of Na^+ content was subsequently accompanied with diminution in K^+ content. Thus, at the highest concentration of NaCl, K^+ concentration decreased by about 51.6, 53.8 and 60.3% of the control in CP59-73 calli and by about 37.4, 46.3 and 55.2% compared to the control in NCo310 ones, respectively after 1, 2 and 3 months of stress exposure (Figure 4).

Consequently, K^+/Na^+ ratio (Table 2) decreased continuously with the NaCl concentration and the duration of stress exposure and this decrease was relatively more important in CP59-73 calli than in NCo310 ones. Ca^{2+} concentration followed almost the same pattern as K^+ concentration and a very significant reduction in Ca^{2+} content in both cultivars stressed calli was observed (Figure 5). Ca^{2+} reduction was lesser in resistant cv. NCo310 calli than in the CP59-73 ones. In presence of 150 Mm of NaCl, Ca^{2+} concentration decreased over time

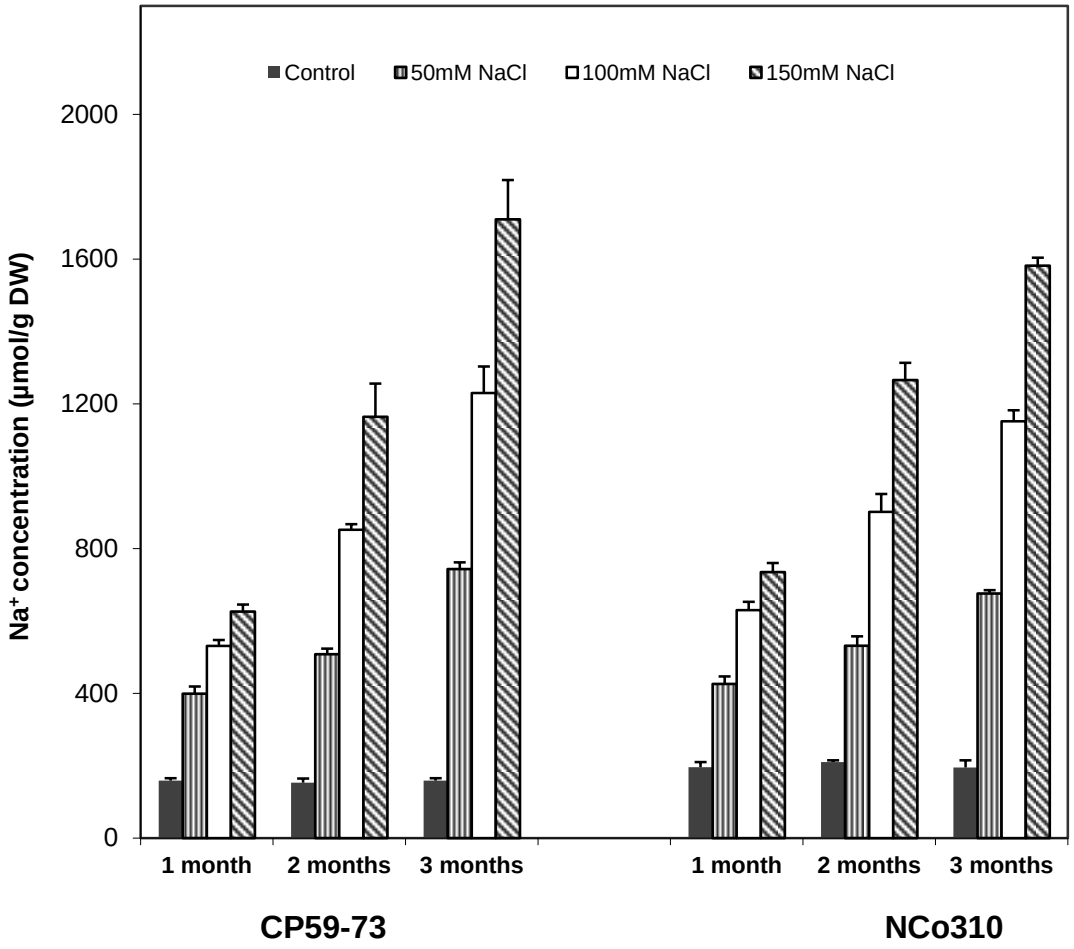

Figure 3. Effect of NaCl induced stress on Na^+ concentration in sugarcane (*Saccharum* sp.) cvs CP59-73 and NCo310 after 1, 2 and 3 months of stress exposure. Vertical bars are means ±SEs, n=5.

and reached to about 57, 62.8 and 67.2% of the control in CP59-73 calli and to about 37.1, 49.3 and 53% of the control in NCo310 ones, respectively after 1, 2 and 3 months of stress exposure.

DISCUSSION

In the present study, the effects of long-term NaCl stress on sugarcane callus growth and ion composition were investigated. The relative growth rate (RGR) varied along the subcultures under stress and non-stress conditions in the culture media. In the absence of stress, RGR decreased significantly among the 1st, the 2nd and the 3rd month of salt stress exposure. This growth reduction could be attributed partially to the high osmotic pressure of the MS basal medium, which might produce an osmotic stress (Rus et al., 2000; Lutts et al., 1996). Under salt stress conditions, the calli obtained from both sugarcane cvs exhibited the same general tendency in response to NaCl concentration and to the duration of salt stress exposure. Thus, RGR decreased considerably in both sugarcane cvs calli. This reduction was continuous over the time and reached very low values after the three months of subcultures in saline media. Similar results were obtain in rice (Lutts et al., 1996; Basu et al., 2002; Rattana and Bunnag, 2015), in borage (Al-Mohammed Maher et al., 2014), in alfalfa (Chaudhary et al., 1997), in safflower (Soheilikhah et al., 2015), in tomato (Rus et al., 2000), in potato (Forooghian and Esfarayeni, 2013) and in other sugarcane cultivars (Gandonou et al., 2005b). The decline in callus growth under salt stress is mainly due to the nutritional imbalance as a result of the interference of the accumulated Na^+ and Cl^- ions with essential nutrients involved in both uptake and translocation processes (Patade et al., 2008). RGR reduction was more drastic in CP59-73 calli than in resistant cv. NCo310 ones, which

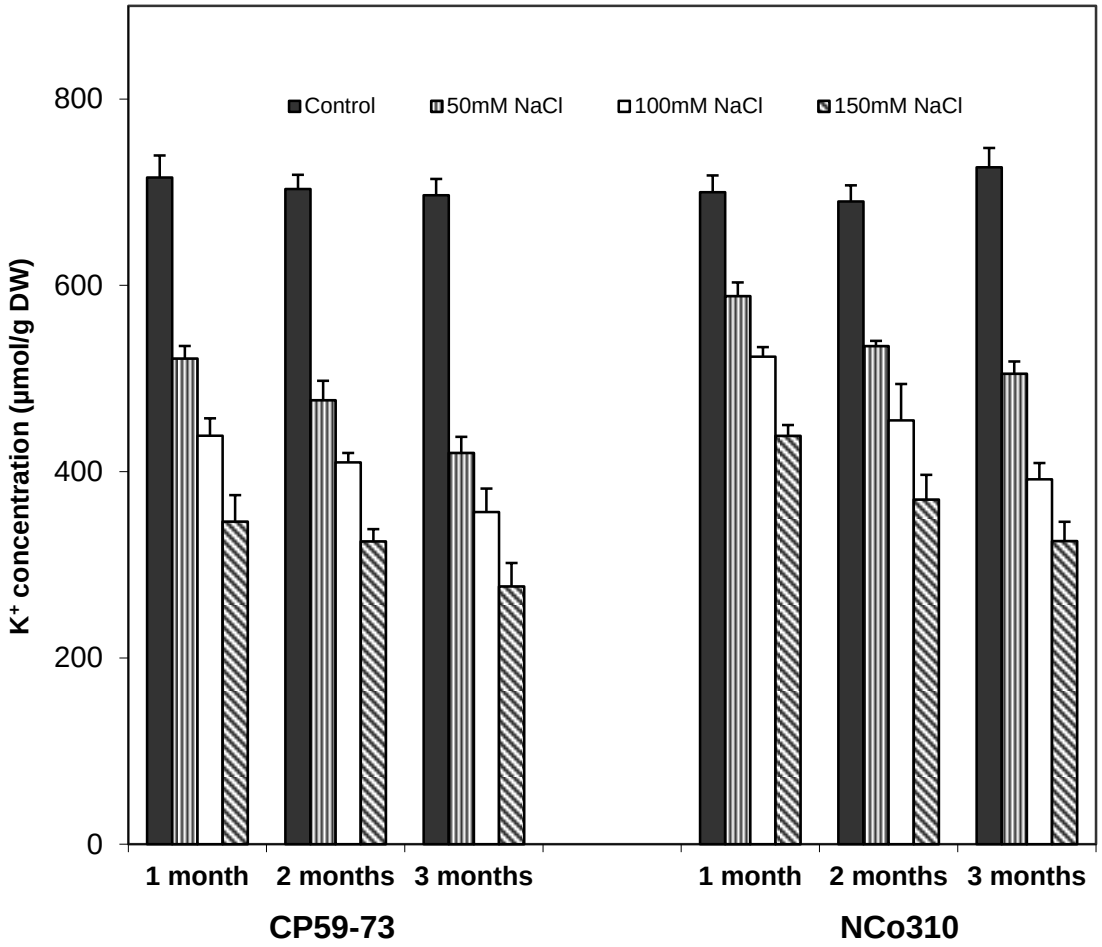

Figure 4. Effect of NaCl induced stress on K^+ concentration in sugarcane (*Saccharum* sp.) cvs CP59-73 and NCo310 after 1, 2 and 3 months of stress exposure. Vertical bars are means ±SEs, n=5.

Table 2. Effect of NaCl induced stress on K^+/Na^+ ratio in sugarcane (*Saccharum* sp.) cvs CP59-73 and NCo310 after 1, 2 and 3 months of stress exposure.

Treatments	K^+/Na^+ ratio					
	CP59-73			NCo310		
	1 month	2 months	3 months	1 month	2 months	3 months
Control	4.5±0.038	4.59±0.25	4.39±0.061	3.58±0.16	3.29±0.17	4.15±0.275
50mM NaCl	1.31±0.032	0.94±0.012	0.57±0.009	1.39±0.03	1±0.047	0.75±0.01
100mM NaCl	0.83±0.01	0.49±0.003	0.29±0.032	0.83±0.01	0.38±0.035	0.34±0.006
150mM NaCl	0.55±0.028	0.27±0.01	0.17±0.045	0.59±0.005	0.29±0.02	0.2±0.01

Values are means ±SEs, n=5.

corroborated our previous conclusions in term of stress tolerance of the studied sugarcane cultivars (Errabii et al., 2007).

The results obtained demonstrated that the intensity and the duration of stress altered continuously and drastically the ion composition in stressed sugarcane calli. In the presence of NaCl, Na^+ concentration increased continuously with the increase of the intensity and the duration of stress in both sugarcane cvs. This response was more important in CP59-73 stressed calli

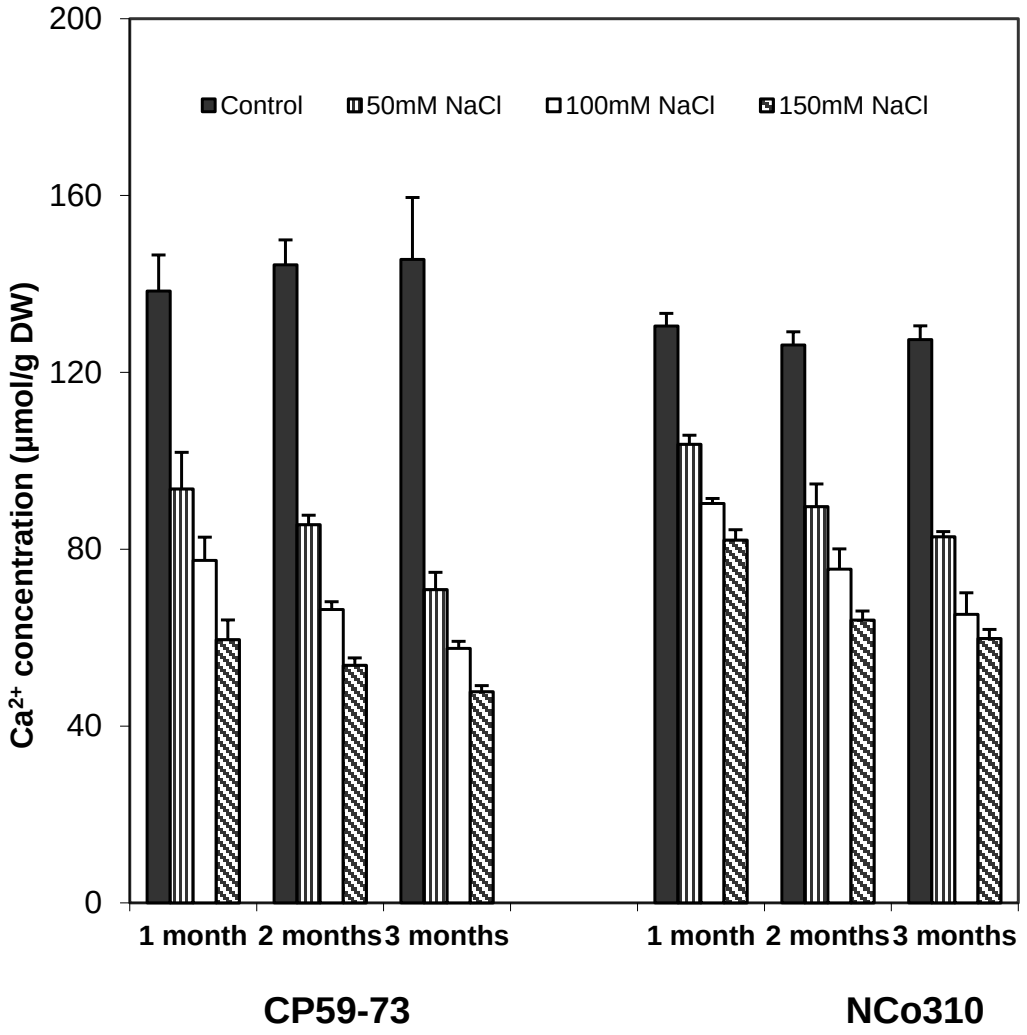

Figure 5. Effect of NaCl induced stress on Ca^{2+} concentration in sugarcane (*Saccharum* sp.) cvs CP59-73 and NCo310 after 1, 2 and 3 months of stress exposure. Vertical bars are means ±SEs, n=5.

than in resistant cv. NCo310 ones. The same tendency was reported in rice calli (Lutts et al., 1996) where the salt-resistant cv. accumulates less Na^+ in comparison with the salt-sensitive cv. However, with other rice varieties, Basu et al. (2002) reported an opposite behavior where calli of the salt tolerant variety SR-26B accumulated more Na^+ than those issued from the salt sensitive Basmati 370. Moreover, in sugarcane, Gandonou et al. (2005a) found no difference in Na^+ accumulation of calli of the salt-resistant cultivar NCo310 and that of the salt-sensitive cultivar CP65-357 and concluded that Na^+ is not directly implied in salt-tolerance of sugarcane at cellular level. It seems not to be the case in this study and according to the results, it is logical to infer that the salt tolerance in sugarcane could be due to the restriction of Na^+ accumulation and to the development of exclusion mechanism that cope with the presence of NaCl in culture medium.

The increase in Na^+ concentration was accompanied with a subsequent decrease in K^+ and Ca^{2+} contents mainly in CP59-73 calli. These results indicate that higher K^+ accumulation can be used as a criterion to discriminate salt-sensitive cultivars in sugarcane, at least at the cellular level. Moreover, the K^+/Na^+ ratio decreased in response to the intensity and the duration of stress. Similar results were previously reported in alfalfa (Chaudhary et al., 1997) and in *Cymbopogon martinii* (Patnaik and Debata, 1997), in soy (Liu and Van Staden, 2001) and in rice (Sathish et al., 1997). Likewise, Sairam et al. (2002) described a similar trend in wheat genotypes in response to long-term salt stress. These findings could be attributed to the substitution of K^+ by Na^+ within the callus cells to achieve the osmotic adjustment since both ions compete for the same binding site as reported by

Lokhande et al. (2010). Moreover, the reduction of K^+/Na^+ ratio within callus cells may disrupt the enzymatic processes, the turgor pressure of the cell and the translocation of fixed carbon leading ultimately to a reduction of calli RGR under salt stress conditions (Marcum et al., 2007; Szczerba et al., 2009).

Calcium is a key component in ion uptake regulation and it promotes the uptake of K^+ versus Na^+ (Hirschi, 2004). This statement could explain the relationship between the drastic diminution of Ca^{2+} and the important accumulation of Na^+ especially in CP59-73 stressed calli. Also, the obtained results demonstrated that stress intensity and duration of stress exposure altered continuously and drastically the ion composition in stressed sugarcane calli. In rice calli, a different behavior was recorded: Cations (Na^+, K^+ and Ca^{2+}) contents alterations occurred only in the first month and it remain constant during the second and the third month of salt stress exposure (Lutts et al., 1996).

The accumulation of Cl^- occurred in stressed calli obtained from both sugarcane cultivars and was proportional to the intensity and duration of stress. These results corroborated with those obtained in *Oryza sativa* (Lutts et al., 1996) and in alfalfa (Chaudhary et al., 1997). In sugarcane, the calli obtained from the salt resistant cv. NCo310 accumulated more Cl^- than those obtained from CP59-73. In fact, the Cl^- accumulation does not cause much injury at the cellular level in sugarcane and it achieves a crucial role in osmotic adjustment after a long-term salt stress exposure (Errabii et al., 2007).

Several authors described the important role of the inorganic fraction in maintaining osmotic adjustment. Short and Colmer (1999) signaled that in halophyte *Halosarcia pergranulata,* Na^+ and Cl^- ensured 80% of osmotic potential. In this work, the real ions contents involved in vacuolar osmotic adjustment are not determined with exactitude since the concentration given here included apoplastic, cytoplasmic and vacuolar ion contents. However, the viability of stressed calli obtained from both sugarcane cvs allowed us to assume that, at the cellular level, the salt-tolerant cultivar responds to elevated NaCl concentrations by an efficient Na^+ and Cl^- compartmentalization so it maintains a low cytosolic Na^+ concentrations and high cytosolic K^+/Na^+ ratios through the extrusion mechanism (Blumwald, 2000).

Salt stress acts both by its intensity and by its duration. In short-term stress exposure, the accumulation of Na^+ and Cl^- in stressed calli might restrict water loss and contributes to osmotic adjustment. As well, the stress tolerance in sugarcane calli seems to be related to maintain of an optimum K^+/Na^+ ratio and an efficient compartmentalization of toxic ions. However, after a long-term stress exposure, the capacity of cells to compartmentalize the ions into the vacuole is exceeded leading to severe ion imbalance and callus growth reduction in sugarcane cvs.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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ABBREVIATIONS

RGR, Relative growth rate; **cv,** cultivar; **MS medium,** Murashige and Skoog medium; **FW,** fresh weight; **DW,** dry weight.

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