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ORIGINAL PAPER

# Selection of callus cultures of sugarcane (*Saccharum* sp.) tolerant to NaCl and their response to salt stress

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**Abstract** Stable callus cultures tolerant to NaCl (68 mM) were developed from salt-sensitive sugarcane cultivar CP65-357 by in vitro selection process. The accumulation of both inorganic ($Na^+$, $Cl^-$ and $K^+$) and organic (proline and soluble sugars) solutes was determined in selected and non-selected calli after a NaCl shock in order to evaluate their implication in in vitro salt tolerance of the selected lines. Both salt-tolerant and non-selected calli showed similar relative fresh weight growth in the absence of NaCl. No growth reduction was observed in salt-tolerant calli while a significant reduction about 32% was observed in nonselected ones when both were cultivated on 68 mM NaCl. Accumulation of $Na^+$ was similar in both salt-tolerant and non-selected calli in the presence of NaCl. Accumulation of $Cl^-$ was lower in NaCl-tolerant than in non-selected calli while proline and soluble sugars were more accumulated in salt-tolerant than in non-selected calli when both were exposed to salt. $K^+$ level decreased more severely in non-selected calli than in NaCl-tolerant ones after NaCl shock. The results indicated that $K^+$ and $Cl^-$ may play a key role in in vitro salt-tolerance in sugarcance cell lines obtained by in vitro selection and that organic solutes could contribute mainly to counteract the negative water potential of the outside medium.

**Keywords** Sugarcane (*Saccharum* sp.) · NaCl tolerance · Inorganic solutes · Organic solutes · In vitro selection

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## Introduction

High concentrations of salts in soils account for large decreases in the yield of a wide variety of crop all over the world (Tester and Davenport 2003). Approximately 5% of the cultivated land is affected by salt (Munns et al. 1999). The understanding of the mechanisms that enable plants to adapt to salt stress is necessary for exploiting saline soils. Salt tolerance in higher plants is the result of numerous physiological and biochemical processes.

Plant tissue culture techniques have been used to produce salt-tolerant cell lines and plants in several species such as wheat (Barakat and Abdel-Latif 1996), rice (Lutts et al. 1999), barley (Sibi and Fakiri 2000), potato (Benavides et al. 2000) and sunflower (Alvarez et al. 2003). This suggests that tissue culture selection is an adequate model to select tolerant clone from overall non-tolerant populations and to research the

adaptative mechanisms of plants living in saline environment. In callus and suspension cultures of potato selected for in vitro NaCl tolerance, Sabbah and Tal (1990) have found that non-electrolytes were the main contributors to the decrease in osmotic potential in both callus and suspension culture. Furthermore, Basu et al. (2002) reported that $K^+$ was the first candidate to counteract the negative water potential of outside medium and that proline appeared to be the last metabolite device that rice calli (adapted to NaCl) opted for when exposed to salt stress.

Although the studies related to the selection and the physiological characterization of cell lines tolerant to salt stress are abundant in the literature, none of them was focused on sugarcane. In our research, we selected salt-tolerant calli of sugarcane in order to study the physiological and biochemical adaptative mechanisms implied in their salt tolerance. The aims of this study were to compare salt-stress effects on growth, $Na^+$, $Cl^-$, $K^+$, proline and soluble sugars accumulation in non-selected and NaCl-tolerant calli.

## Materials and methods

### Plant material

Stalk segments of sugarcane variety CP65-357 (salt-sensitive) were sterilized with ethanol 70% and sown in pots containing sand in greenhouse. After bud emergency, sugarcane plants were grown in these conditions until approximately 7 months.

### Callus induction and in vitro selection

Callus induction was made on Murashige and Skoog medium containing 30 g $l^{-1}$ sucrose, 8.0 g $l^{-1}$ agar and 3 mg $l^{-1}$ 2,4 dichlorophenoxyacetic acid. The medium was adjusted to pH 5.8 with NaOH (0.1 N), autoclaved at 120°C and 1 bar for 20 min and dispensed in jar (30 ml in each jar). Calli were initiated from young leaf explants and incubated at 25 ± 1°C in darkness in growth cabinet according to our previous study (Gandonou et al. 2005a). After 4 weeks, calli were separated from the explant and subcultivated to fresh media for 4 other weeks for further proliferation. Calli were then subcultivated in petri dishes on MS medium described above (25 ml per Petri dish), added with 68 mM NaCl to initiate a shock selection. This concentration of NaCl was chosen following our previous report which revealed that 68 mM of NaCl decreased significantly CP65-357 callus growth (Gandonou et al. 2005a). Twenty calli were used for both control (non-saline) and selection (saline) media. After 16 weeks (4 × 4 weeks), most of the calli cultivated on 68 mM NaCl medium became brown except for small groups of cells that remained light in colour. These sectors were transferred to the same medium for 8 weeks (2 × 4 weeks) for further proliferation after what they were transferred to non-saline medium for 8 weeks (2 × 4 weeks) for stabilization. After that, calli were transferred again to the selection medium (68 mM NaCl) for 16 weeks (4 × 4 weeks). Control (non-selected) calli were continuously cultivated in MS medium without NaCl.

The assay was initiated according to the following scheme: (1) calli from the control medium were transferred either to non-saline medium, or to medium with 68 mM NaCl added; (2) calli from the selected line were transferred to either non-saline medium or 68 mM NaCl added medium.

### Callus growth determination

The fresh weight of control and salt-adapted calli was determined at 0 time ($W_0$) and 5 weeks after the beginning of the experiment ($W_f$) on media containing 0 or 68 mM NaCl to estimate the extend of adaptation achieved by selected tissues. Relative fresh weight growth rate (RFWG) of calli was calculated as $(W_f - W_0)/W_0$.

### Extraction and estimation of ion concentrations

For ions determination, calli were rinsed for 5 min in cold distilled water in order to remove a maximum solute present in apoplasm without a substantial elimination of cytosolic solutes as

recommended (Sacchi et al. 1995). Calli were oven-dried at 80°C for 48 h and ground in a poter and the powder was dried for 24 h. $Na^+$, $K^+$ and $Cl^-$ determination was done as reported in our previous report (Gandonou et al. 2005b). Ions concentrations are expressed in dried matter basis with NaCl ($Na^+$ and $Cl^-$) and KCl ($K^+$) as standard.

### Extraction and proline determination

Proline extraction and determination were done as reported in our previous report (Gandonou et al. 2005b) with 50 mg of tissue per callus.

### Extraction and soluble sugars determination

For soluble sugars determination, 50 mg of tissue per callus were ground in a mortar, homogenised in 1 ml of ethanol 80% and centrifuged at 5000*g* for 10 min at 4°C. Supernatants were transferred in other tubes and the pellets were homogenized again in 0.5 ml ethanol 80% and centrifuged as above. The second supernatant was added to the first. Total soluble sugars were measured by a modified method of Watanabe et al. (2000). One milliliter of extract was reacted with 3 ml freshly prepared anthrone reagent (50 mg anthrone + 50 ml of $H_2SO_4$ 95%) at 100°C for 10 min. After cooling in ice, the total sugar content was determined at 620 nm by a spectrophotometer using glucose as standard.

### Statistical analysis

For each experiment, 20 calli were used (5 per jar). Ten calli were used for growth determination in each treatment except in the case of non-adapted calli cultivated in 68 mM NaCl where 15 calli were used because of eventual callus necrosis. Each value is presented in the form of mean ± standard error with a reading of four (growth, $Na^+$, $K^+$, proline and soluble sugars) or three ($Cl^-$ content) samples per treatment. The analysis of the main effects of cultivars and stress was based on a 2-ways analysis of variance (ANOVA). All statistical analyses were performed by SAS 92 program (SAS Institute 1992).

**Table 1** Results of 2-ways variance analysis for RFWG, ion content, proline and soluble sugars accumulation of non-selected and NaCl-tolerant sugarcane calli

| Parameter | Stress | Type of callus | Interaction (stress × type of callus) |
|---|---|---|---|
| RFWG | 42.63*** | 22.84*** | 34.80*** |
| $Na^+$ | 50.42*** | $0.55^{ns}$ | $0.10^{ns}$ |
| $K^+$ | $2.87^{ns}$ | $0.16^{ns}$ | $0.31^{ns}$ |
| $Cl^-$ | 53.01*** | $0.11^{ns}$ | 8.60* |
| Proline | 20.414*** | 8.05* | $0.28^{ns}$ |
| Soluble sugars | 10.05** | 19.25*** | $1.79^{ns}$ |

*F*-ratios are given for the main effects of the following levels of classification: stress (i.e. presence of NaCl in the media), type of calli and interaction between these levels of classification (ns, not-significant; *significant at $P = 0.05$; **significant at $P = 0.01$; ***significant at $P = 0.001$)

## Results

### Growth

In the absence of stress, RFWG was similar for both selected and non-selected calli. Salt effect results in a significant reduction in non-selected calli RFWG ($P < 0.001$, Table 1) while no significant reduction was observed in selected ones (Fig. 1). In the case of non-selected calli, RFWG decreased from 1.318 in the absence of stress to 0.899 at 68 mM NaCl after 5 weeks; this decrease corresponded to 32%. RFWG of selected calli was about 1.280 in the absence of stress and about 1.259 after 5 weeks at 68 mM NaCl. The significant

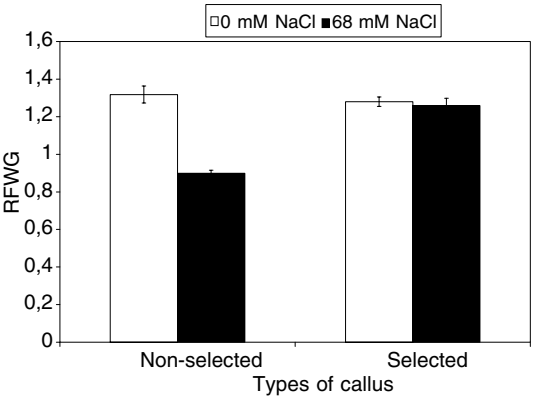


**Fig. 1** Relative fresh weight growth (RFWG) of non-selected and NaCl-tolerant (to 68 mM NaCl) sugarcane on non-saline or saline (68 mM NaCl) media after 5 weeks of culture ($n = 4$): vertical bars are standard errors

difference ($P < 0.001$, Table 1) observed between the types of calli in their growth indicated that selected calli grew faster than non-selected calli when both were cultivated in the presence of NaCl.

## Ion content

In the absence of stress, non-selected and selected calli did not differ in $Na^+$ and $K^+$ contents but $Cl^-$ content in selected calli was higher than that of non-selected calli (Fig. 2a–c). A significant increase ($P < 0.001$, Table 1) in $Na^+$ and $Cl^-$ contents and a decrease (non-significant) in $K^+$ content were recorded in the presence of NaCl in both types of calli (Fig. 2a–c). From 4.39 mg $g^{-1}$ dm in the absence of stress, it reached 11.58 mg $g^{-1}$ dm at 68 mM NaCl in non-selected calli; for selected calli, it passes from 4.10 mg $g^{-1}$ dm in absence of stress to 10.46 mg $g^{-1}$ dm at 68 mM NaCl (Fig. 2a). Both types of calli accumulated similar quantities of $Na^+$ when exposed to NaCl. In response to NaCl, $Cl^-$ content increased significantly ($P < 0.001$, Table 1) in the both types of calli but the accumulation was lower in selected calli than non-selected ones. From 4.52 mg $g^{-1}$ dm in the absence of stress, $Cl^-$ content reached 19.71 mg $g^{-1}$ dm at 68 mM NaCl for non-selected calli while it increased from 8.40 mg $g^{-1}$ dm in the absence of stress to 14.87 mg $g^{-1}$ dm at 68 mM NaCl in the case of selected calli (Fig. 2b). The significant interaction ($P < 0.05$, Table 1) observed between NaCl concentration and the type of calli indicated that selected calli presented $Cl^-$ content higher than that of non-selected calli in the absence of salt and the opposite trend was observed in the presence of NaCl. Thus selected calli accumulated less $Cl^-$ than non-selected calli when both were exposed to the same concentration of NaCl.

$K^+$ concentration decreased under salt stress in the case of non-selected calli but no decrease was observed in the case of selected calli. From

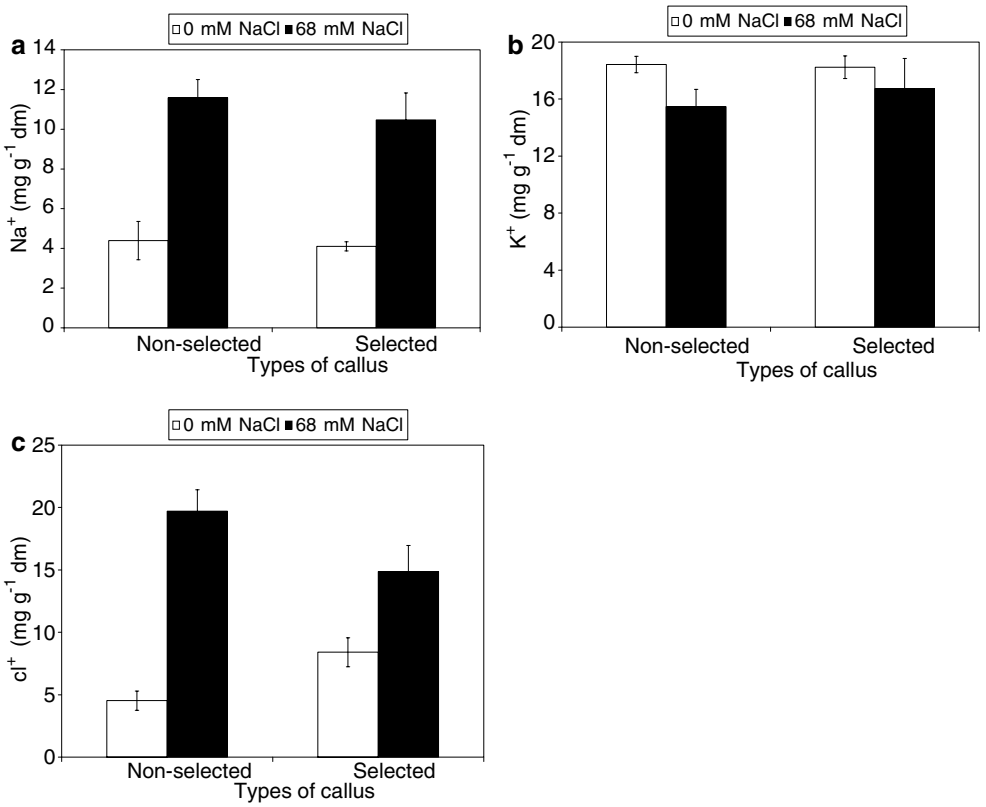


**Fig. 2** (**a**) $Na^+$ content [mg $g^{-1}$ (dm)] ($n = 4$), (**b**) $K^+$ content [mg $g^{-1}$ (dm)] ($n = 4$), (**c**) $Cl^-$ content [mg $g^{-1}$ (dm)] ($n = 3$) of non-selected and NaCl-tolerant (to 68 mM NaCl) sugarcane on non-saline or saline (68 mM NaCl) media after 5 weeks of culture: vertical bars are standard errors

18.42 mg $g^{-1}$ dm in the absence of stress, it reached 15.45 mg $g^{-1}$ dm for non-selected calli while it passes from 18.23 mg $g^{-1}$ dm in the absence of NaCl to 16.72 mg $g^{-1}$ dm at 68 mM NaCl in the case of selected calli (Fig. 2c). The reduction corresponded, respectively, to 16.2% and 8.3%; however, the decrease is not significant either in the case of non-selected or in selected calli (Table 1).

## Organic solutes accumulation

Proline and soluble sugars contents were higher in selected calli than in non-selected calli in the absence of stress (Fig. 3a, b). In the presence of NaCl, free proline contents increased significantly in both non-selected and selected calli ($P < 0.001$, Table 1) and this increase was more accentuated in selected calli in comparison with non-selected calli (Fig. 3a). From 12.15 μmol $g^{-1}$ fm in the absence of stress, proline content reached 18.6 μmol $g^{-1}$ fm at 68 mM NaCl in the case of non-selected calli while it increased from 15.4 μmol $g^{-1}$ fm in the absence of NaCl to 26.15 μmol $g^{-1}$ fm at 68 mM for selected calli (Fig. 3a). So selected calli accumulated significantly ($P < 0.05$, Table 1) more proline than non-selected calli when both were exposed to the same concentration of NaCl.

Similar trends were observed in total soluble sugars profile; sugars content increased significantly ($P < 0.01$, Table 1) from 22.8 mg $g^{-1}$ fm in the absence of stress to 26.37 mg $g^{-1}$ fm at 68 mM NaCl in the case of non-selected calli while it increased from 28.75 mg $g^{-1}$ fm in the absence of stress to 37.54 mg $g^{-1}$ fm at 68 mM NaCl for selected calli (Fig. 3b). So selected calli accumulated significantly ($P < 0.001$, Table 1) more soluble sugars than non-selected ones either in the presence or in the absence of NaCl.

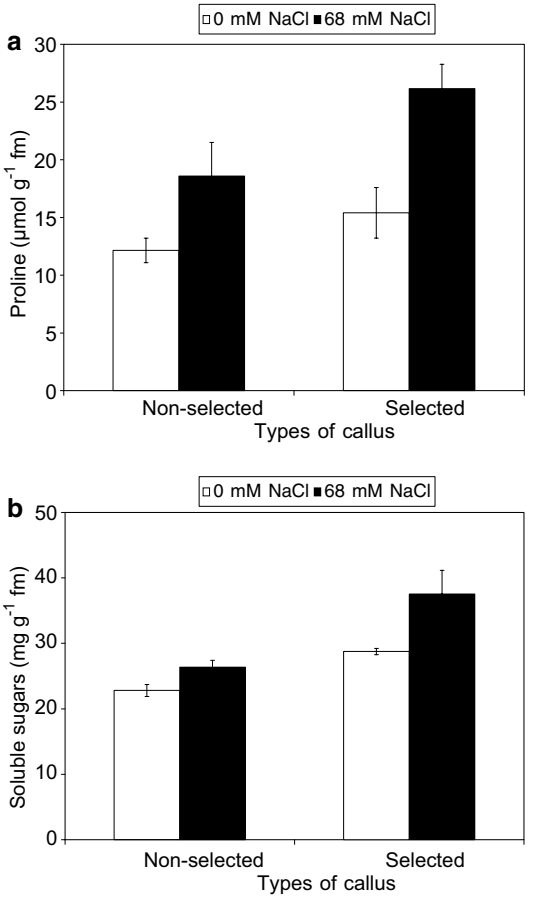


**Fig. 3** (**a**) Proline content [μmol $g^{-1}$ (fm)], (**b**) Soluble sugars content [mg $g^{-1}$ (fm)] of non-selected and NaCl-tolerant (to 68 mM NaCl) sugarcane on non-saline or saline (68 mM NaCl) media after 5 weeks of culture ($n = 4$): vertical bars are standard errors

## Discussion

Many studies in the recent years have tried to develop salt-resistant plants through the use of tissue and cell culture. The first step of these methods was the selection of cell lines exhibiting enhanced tolerance to salinity. Salt-tolerant cell lines have been developed in several glycophytic species such as alfalfa (Croughan et al. 1978), potato (Sabbah and Tal 1990), wheat (Piri et al. 1994), tomato (Kripkyy et al. 2001), rice (Basu et al. 2002) sunflower (Davenport et al. 2003), and *Catharanthus roseus* (Elkahoui et al. 2005).

We developed NaCl-tolerant sugarcane calli using in vitro selection techniques. Sustained growth of selected calli in 68 mM NaCl medium indicated that the tissues were tolerant as reported in rice (Basu et al. 2002). These authors reported that rice calli selected for salt tolerance maintained high viability and regrowth capacity in the presence of NaCl in comparison with the non-selected calli. Our results were in agreement with those reported

in *Catharanthus roseus* suspensions cells adapted to 50 mM NaCl (Elkahoui et al. 2005).

A long-term culture in 68 mM NaCl medium enabled the tissue to accumulate more $Cl^-$ in comparison with non-selected tissue while both selected and non-selected tissues accumulated same quantity of $Na^+$ in NaCl free medium. This suggests that gradual adaptation of tissue in response to repeated culture in saline medium is directly related to uptake of $Cl^-$. Selected calli accumulated more proline and soluble sugars than non-selected calli in the absence of stress. Perhaps $Cl^-$ ions were sequestrated in vacuoles and organic solutes accumulated in cytoplasm as osmolytes.

When cultured in 68 mM NaCl medium, non-selected and selected calli accumulated similar quantity on $Na^+$. This result indicated that $Na^+$ toxicity is not directly implicated in in vitro salt tolerance in sugarcane as reported in our previous report (Gandonou et al. 2005b). Unlike $Na^+$, $Cl^-$ was more accumulated in non-selected calli than in NaCl-adapted calli indicating that $Cl^-$ toxicity may be the main part of salt effect on sugarcane calli; thus the salt tolerance of selected calli may be due to their ability to limit tissue accumulation of $Cl^-$. We have reported that $Cl^-$ ions played a key role in sugarcane in vitro salt tolerance and that calli of the salt-tolerant cultivar NCo310 accumulated more $Cl^-$ than that of the salt-sensitive CP65-357 (Gandonou et al. 2005b) but the fact that the selected calli accumulated less $Cl^-$ than non-selected ones when both were cultivated in the same NaCl medium suggests that in vitro selection techniques could allow salt-tolerant mechanisms different to those occurred naturally in tissue. It was found that salt-adapted and non-adapted suspension cells of *Catharanthus roseus* accumulated same quantity of $Na^+$ and $Cl^-$ ions when both were cultivated on 50 mM NaCl medium (Elkahoui et al. 2005).

Potassium $K^+$ is known to play a main role in osmotic adjustment during stress (Wu et al. 1996). Selected calli maintained higher $K^+$ concentration than non-selected ones when both were cultivated in 68 mM NaCl medium indicating that the salt tolerance of the first was related to their capacity to maintain high $K^+$ content in the presence of the excess of $Na^+$. These results were in agreement with those reported in rice (Basu et al. 2002). We have also found that calli of the in vitro salt-tolerant sugarcane cultivar NCo310 maintained high level of $K^+$ in comparison with that of the salt-sensitive CP65-357 when both were cultivated in the presence of NaCl (Gandonou et al. 2005b).

Proline accumulation was higher in selected calli than in non-selected ones either in the absence of NaCl or in its presence. Proline accumulation was frequently reported in salt-stressed calli and whole plants. Most usually, it was considered to act as a compatible osmoticum and therefore to be involved in salt-resistance mechanisms (Delauney and Verma 1993; Alvarez et al. 2003; Ehsanpour and Fatahian 2003; Misra and Gupta 2005). In rice, Lutts et al. (1996) reported that proline appeared to be a symptom of injury rather than indicator of salinity resistance since the salt-sensitive cultivar accumulated more proline than the tolerant one when both were cultivated in the presence of NaCl. Our present results indicated that proline may play a significant role in in vitro salt tolerance in sugarcane calli obtained by in vitro selection process. In our previous report, we have found that proline was not implied in sugarcane in vitro salt tolerance using two cultivars differing in their salt tolerance (Gandonou et al. 2005b). This difference between the behaviour of calli issued from cultivars and in vitro selected calli could be explained by the fact that in vitro selection process may imply mechanisms different from those implied in natural salt tolerance. Gangopadhyay et al. (1997) also reported that a salt-adapted calli of tobacco retained more proline than that of unadapted calli when both were treated with salt shock. So proline appeared to be responsible, at least partially, for imparting salt tolerance to the tissue of sugarcane NaCl-tolerant calli.

Soluble sugars accumulation under salt stress was reported in many species such as tomato (Bourgeais-Chaillou and Guerrier 1992) or *Populus euphratica* (Watanabe et al. 2000) where they seemed to be implied in osmotic adjustment. We found that salt-tolerant calli accumulated more soluble sugars than non-selected ones. Our results were not in agreement with those reported in tomato Perez-Alfocea et al. (1994); they found that soluble sugars content increased significantly

in calli issued from salt-sensitive species while a significant decrease (50%) was observed in calli issued from the salt-tolerant species indicating that soluble sugars were not implied in salt tolerance in these species. Our findings that these solutes were more accumulated in selected calli either in non-saline and saline conditions indicated that soluble sugars may play an important role in salt tolerance of selected calli.

## Conclusion

This work demonstrates that in vitro selection techniques can be used to generate salt-tolerant cell lines in sugarcane and to study physiological and biochemical indicators of salinity tolerance in this plant. Thus salt tolerance seemed to be related to the efficiency of a tissue to modulate the level of inorganic and organic solutes in response to salt stress. These results indicate that $Cl^-$ exclusion combined to $K^+$, proline and soluble sugars accumulation are the main option to counteract the negative effects of salt stress in sugarcane selected calli.

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