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Adaptation of sugarcane plants to saline soil

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Highlights

- Genetic variation in salt tolerance was found within sugarcane
- The salt-tolerant cultivar maintained biomass, leaf area and photosynthesis at 160 mM NaCl, whereas the salt-sensitive cultivar suffered at 80 mM NaCl
- The salt-tolerant cultivar excluded Na^+ from roots and leaves
- Proline increased with increasing salinity but glycine betaine did not

Abstract

Sugarcane is an important crop in tropical regions of the world, often being exposed to environments with high salinity, but little is known of genetic variation in salt tolerance. The aim of this work was to compare the performance of two genetically diverse cultivars of sugarcane under different concentrations of salinity (0, 40, 80 and 160 mM NaCl) over a period of 30 days. SP 81-3250 was more salt-tolerant and maintained its rate of biomass production, photosynthesis and leaf area up to 160 mM NaCl, whereas IAC 87-3396 was sensitive to 80 mM NaCl. SP 81-3250 maintained very low concentrations of Na^+ in both leaves and roots with increasing time and salinity, whereas in IAC 87-3396 the Na^+ concentrations were 2-5 times higher. SP 81-3250 had a greater accumulation of proline, and lower lipid peroxidation, whereas glycine betaine and sucrose concentrations were similar in the two cultivars. This

suggests that the tolerance of SP 81-3250 to high salinity was due to its ability to exclude Na^+ while taking up water from the soil, and that measurements of Na^+ concentration in leaves could be used to select salt-tolerant genotypes for saline areas.

Keywords: Gas exchange; water potential; sodium; potassium; proline; glycine betaine.

1. Introduction

Most crops suffer a significant reduction in biomass when soils become saline, so the extent of salinized soils worldwide has become a serious problem for productive, quality and sustainable agriculture. In order to increase food production and to supply the needs of a growing population in the world, agriculture has expanded into soils that are salinized, either by their geological origin in arid and semi-arid regions or by management practices of land clearing or irrigation (Pitman and Läuchli, 2000). Salinity is also a problem in tropical regions from encroachment of seawater into low-lying coastal areas. Approximately 7% of the total land surface incurs soil salinization to some degree (Pessarakli and Szabolcs, 1999).

Sugarcane (*Saccharum* spp.) is an important crop for sugar and biofuel production, and has been cultivated in all tropical and subtropical areas of the world. Brazil is the world's largest sugarcane producer and stands out in the commercialization and export of sugar and ethanol from this crop. Sugarcane is considered as moderately salinity sensitive, and it is estimated that one million hectares of sugarcane cultivation are salinized (Patade et al., 2011). Sugarcane needs soils with high water content and nutrients in order to reach maximum yield, so cultivation in salinized soils can result in a drastic reduction of growth, with losses of 50% or more in comparison to the productivity in unsalted soils (Suprasanna et al., 2011; Sengar et al., 2013; Kumar et al., 2014).

Most plants are susceptible to salt stress and unable to yield well in soil salt concentrations above 100 mM NaCl (Munns and Tester, 2008). Salt tolerance is a complex mechanism, involving tolerance to both the osmotic and ionic stress caused by high soil salinity, and plant species differ in biochemical and physiological responses. Differences also occur within species, especially those species that have high genetic variability, as in sugarcane, and have not undergone persistent selection pressure for individual traits for salt tolerance.

In plants under salinity, both osmotic and ionic effects occur as the high soil salt concentration decreases water uptake, increases salt uptake, and increases cellular

concentrations of Na^+ and Cl^- (Deinlein et al., 2014; Munns and Gilliham, 2015; Parihar et al., 2015; Yousuf et al., 2017). Moreover, salinity stress leads to oxidative stress, to reactive oxygen species (ROS) production and to the limitation of many physiological processes fundamental to plant productivity (Gupta and Huang, 2014; Negrão et al., 2017). Studies on the biochemical and physiological processes fundamental to plant productivity are important to provide a way to mitigate this plant stress. Such studies can identify key functions and provide new genetic sources of salt tolerance for plant breeding (Mudgal et al., 2010; Cabello et al., 2014; Marco et al., 2015; Yousuf et al., 2017).

The regulation of Na^+ and Cl^- uptake is essential in order to avoid toxic Na^+ accumulation in leaves, and to maintain a high K^+/Na^+ ratio which is important for the activity of K^+-dependent enzymes (Shabala and Pottosin, 2014). Na^+ exclusion from leaves has been shown to promote tolerance in salt-sensitive species like wheat and rice (Munns and Gilliham, 2015). The accumulation of certain organic solutes (compatible osmolytes) are some of the biochemical and physiological mechanisms that can enable plants to tolerate stress effects. The amino acid proline (Pro) and the quaternary amine glycine betaine (GB) are organic solutes considered to be biochemical indicators of plant stress, and accumulate in plant tissues to protect against toxic accumulation of salt and promote osmotic adjustment of the cytoplasm in response to various stresses including salinity (Kishor et al., 2014; Rejeb et al., 2014; Singh et al., 2015). In severe osmotic stresses, Pro and GB when accumulated lead to plant osmotic adjustment, cellular turgor recovery, and aid growth in ionic stress (Roychoudhury and Banerjee, 2016). Pro functions as an osmoprotectant involving the stabilization of antioxidant enzymes and other proteins, cellular homeostasis balance and signaling (Liang et al., 2013; Rejeb et al., 2014). GB acting as an osmoprotectant against stress has a special function in chloroplasts and interacts with protein complexes such as Rubisco and protects thylakoid membranes to maintain photosynthetic photochemical efficiency (Chen and Murata, 2011). Moreover, studies have shown that Pro and GB participate in ROS elimination produced in oxidative stress and, therefore, have non-enzymatic antioxidant activity (Signorelli et al., 2015).

Sugarcane has been the subject of physiological studies carried out with different cultivars under salinity conditions, which have been contributed to an understanding of the metabolites and genes that regulate salt tolerance. Saxena et al. (2010) found different sensitivities for different sugarcane cultivars under salt stress, ranging from 6 to 36 % loss of leaf area. Those authors observed increase of protein concentration in stressed plants which

they associated with the plant ability to produce "stress proteins" for tolerance to salinity. Kumar et al. (2014) observed more tolerance to salinity in transgenic sugarcane plants when transformed with *Arabidopsis* Vacuolar Pump (AVP1) gene. Poonsawat et al. (2015) verified that genes like sugarcane shaggy-like kinase (*SuSK*), sucrose transporter (*SUT*), proline biosynthesis (pyrolline-5-carboxylate synthetase; *P5CS*), Na^+/H^+ exchanger for ion homeostasis (*NHX1*), and catalase (*CAT2*) showed higher expression in sugarcane stressed by salinity, contributing to tolerance of those plants by Na^+ reduction, soluble sugars enrichment and increase of free proline. Satbhai and Naik (2014) showed that tolerance to salinity in different sugarcane cultivars was related to better ability for ROS scavenging, maintaining low lipid peroxidation and higher membrane stability, as well as compatible osmolytes. The ability of plants to produce antioxidants enzymes under stress is a well-known mechanism of tolerance. In sugarcane, Patade et al. (2011) found a doubling of the activity of the antioxidants catalase and ascorbate peroxidase with a smaller increase in superoxide dismutase in plants treated with 150 mM NaCl. Besides that, some molecular markers can be useful to discern between susceptible and tolerant cultivars; Markad et al. (2014) showed that ISSR primers were useful to identify tolerant and susceptible cultivars of sugarcane for breeding programs..

This study was carried out with two sugarcane genotypes (SP 81-3250 and IAC 87-3396), with significant productivity in Brazilian soil, based on the hypothesis that the more productive genotype under soil NaCl concentrations presents higher organic solutes accumulation (Pro and GB), and lower H_2O_2 and MDA content. Accumulation of Na^+ and K^+ were also measured. Productivity was assessed as biomass and leaf area production along with photosynthetic activity.

2. Material and methods

2.1 Growth conditions and salt treatments

The experiments were conducted at the Universidade Estadual Paulista, Campus of Jaboticabal, São Paulo, in a greenhouse with natural light, a temperature of 30°C and a relative humidity of 40% (Figure 1A). The two sugarcane cultivars, SP 81-3250 and IAC 87-3396, were obtained from mini-totes provided by the APTA - São Paulo Agribusiness Technology Agency. These two cultivars were chosen because SP 81-3250 is one of the most widely cultivated in Brazil and IAC 87-3396 was recently launched as a plant tolerant to some adverse environmental conditions, such as low available nutrient and water deficit. Their sensitivity to salt stress was unknown.

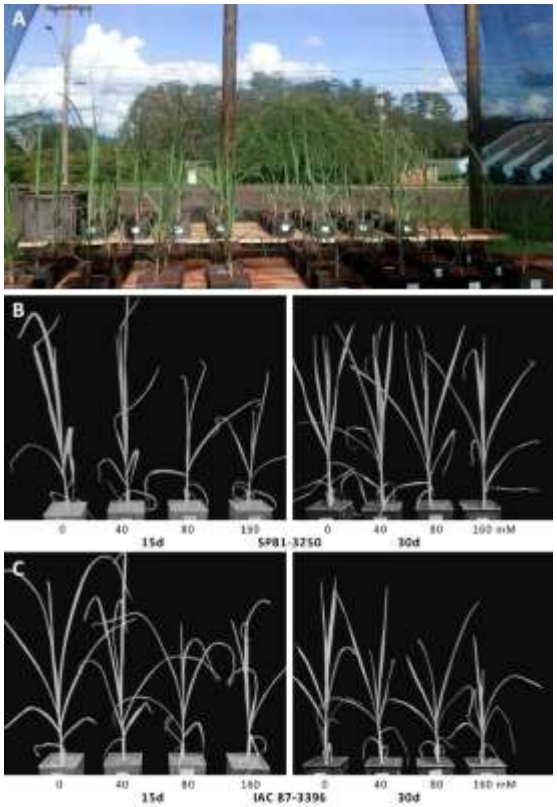


Figure 1. Installation and conditions of main experiment. A: Development of sugarcane plants in greenhouse. B: Plants of SP 81-3250 at 15 and 30 d under salinity levels shown. C: Plants of IAC 87-3396 at 15 and 30 d under salinity levels.

The main experiment was a completely randomized 2x4 factorial scheme (2 cultivars and 4 treatments: 0, 40, 80 and 160 mM NaCl), with four replicates per treatment. The plants from mini-totes were transplanted in 2L pots filled with soil. Soil Dystrophic Red Latosol was collected at 0-20 cm soil depth, with chemical characterization of 5.7 pH; 10 mg dm^{-3} of organic matter, 0.1 mg kg^{-1} of Na^{+}; 26 mg dm^{-3} of P (resin); 1.6 mmol dm^{-3} of K; 34 mmol dm^{-3} of Ca; 21 mmol dm^{-3} of Mg; 341 g $kg^{-1}$of clay; 28 g kg^{-1} of silt; 310 g kg^{-1} of fine sand; 321 g kg^{-1} of coarse sand; mean textural density and density of 1.24 kg dm^{-3}. Soil fertilization was carried out in three stages every 10 days, using 0.3 g of $NH_4H_2PO_4$ and 0.35 g of KH_2PO_4 per pot (incorporated to soil before transplanting) and 0.45 g of urea and 0.35 g of K_2SO_4 (second and third stage, overlay) and the irrigation was performed daily, in order to restore the water loss through evapotranspiration. At 30 d, the salt concentrations in the soil solution was increased to 40, 80 and 160 mM NaCl and the evaluations were performed after 15 and 30 d of treatment. At both harvests, biometric measurements were made on roots and shoots. Leaves and roots were analysed for Na^{+}, K^{+}, proline, and glycine betaine. Leaves were measured for

water potential, malondialdehyde (MDA) content and hydrogen peroxide (H_2O_2), chlorophyll content and quantum yield of PSII.

As well as this main experiment, a preliminary experiment was made using the same sugarcane cultivars (SP 81-3250 and IAC 87-3396) grown under the same conditions as above. At 60 d (30 d of development and 30 d of salinity), gas exchange, stomatal conductance, transpiration, intracellular CO_2, shoot biomass and Na^+ concentration of leaves were evaluated.

2.2 Biometric measurements

Height of the plants was measured from soil surface to the tip of the longest fully expanded leaf. Leaf area and root density were measured after separation of the vegetative parts using the Delta-T Devices LTD image analysis system. The total dry biomass of shoot and root was obtained after drying in a forced ventilation oven at temperature from 65 ° C to constant weight.

Biochemical and physiological evaluations were performed on +1, +2 and +3 leaves, active photosynthetically, according to Kuijper (1915). The +1 leaf is designated by first leaf from top to bottom of the stalk with clearly visible dewlap.

2.3 Tissue ion concentrations

Na^+ and K^+ concentrations in leaves and roots of the two sugarcane cultivars were determined by flame photometry after digestion in HNO_3 according to Bloise and Moreira (1976), using +3 leaves or the whole root system.

2.4 Proline, glycine betaine and sucrose

Proline was determined on leaf (+2 leaves) and root tissues (200 mg) by the method described by Bates et al. (1973). Leaf samples were homogenized in aqueous sulfosalicylic acid (3% w/ v) and the filtered homogenate was reacted with acid ninhydrin at 100ºC for 1 hr. The reaction mixture was extracted with toluene and the absorbance at 520 nm was recorded using toluene as a blank.

Glycine betaine was measured on leaf (+2 leaves) and root tissue (500 mg) by shaking with 20 ml of deionized water for 48 h at 25 °C and extracting with cold potassium iodide-iodine reagent in 1 N sulphuric acid according to Grieve and Grattan (1983). The periodate crystals were dissolved in 1, 2-dichloro ethane and, the absorbance was measured at 365 nm.

Sucrose was measured on 50 mg of lyophilized material (+2 leaves) powder extracted with MCW (methanol, chloroform and water in the proportion of 12:5:3 (v/v)) at 25ºC for 30 min, and centrifuged at 10,000 g for 10 minutes at 20ºC. The residue was re-extracted and the supernatants combined. Sucrose quantification was carried out at 620 nm by the anthrone method of Van Handel (1968), using sucrose as standard.

2.5 Lipid peroxidation: MDA and H_2O_2

Lipid peroxidation was performed in +3 leaves (250 mg) by the 2-thiobarbituric acid (TB) test. The content of the reactive substances (TBARS) was quantified as the final product of the process of lipid peroxidation, with readings at 535 and 600 nm. Malondialdehyde (MDA) content was calculated according to Mihara et al. (1980) and H_2O_2 content was determined according to Gay et al. (1999). The readings of MDA and H_2O_2 the standard curve and the samples were read by a spectrophotometer at 560 nm.

2.6 Leaf water potential (Ψw), chlorophyll (SPAD value) and Fv/Fm

Leaf *Ψw* was determined according to Scholander et al. (1956), using a pressure chamber model m670 - PMS Instrument Co., Albany, USA, (+2 leaves), between 04:30 and 05:30.

Chlorophyll concentration was measured on +1 leaves with a SPAD-502 Chlorophyll Meter (Soil and Plant Analysis Development), Minolta Co.

PSII quantum yield (Fv/Fm) was measured *in vivo* after the leaves were dark adapted for half an hour using a portable fluorometer, model OS-30p (Opti-Science, USA), on +1 leaves. Values for statistical analysis were derived from an average of three readings taken for each pot.

2.7 Gas exchange: A, g_s, E and C_i

Gas exchange was performed using a portable infrared gas analyzer (IRGA), SD-ADC biotechnology model LCpro (Serial System 33726) with an LED light source (blue and red) in a light intensity of 2,000 µmol m^{-2} s^{-1}. Chamber temperature was kept constant at around 26-27 °C. Atmospheric CO_2 concentration was used. Leaves were exposed to light intensity for the stabilization of g_s and E. Three measurements were taken on +1 leaves for each plant, every 30 seconds, after stabilization. Measurements were performed between 09:30 and 11:30, and the mean for A, g_s, E and C_i was calculated.

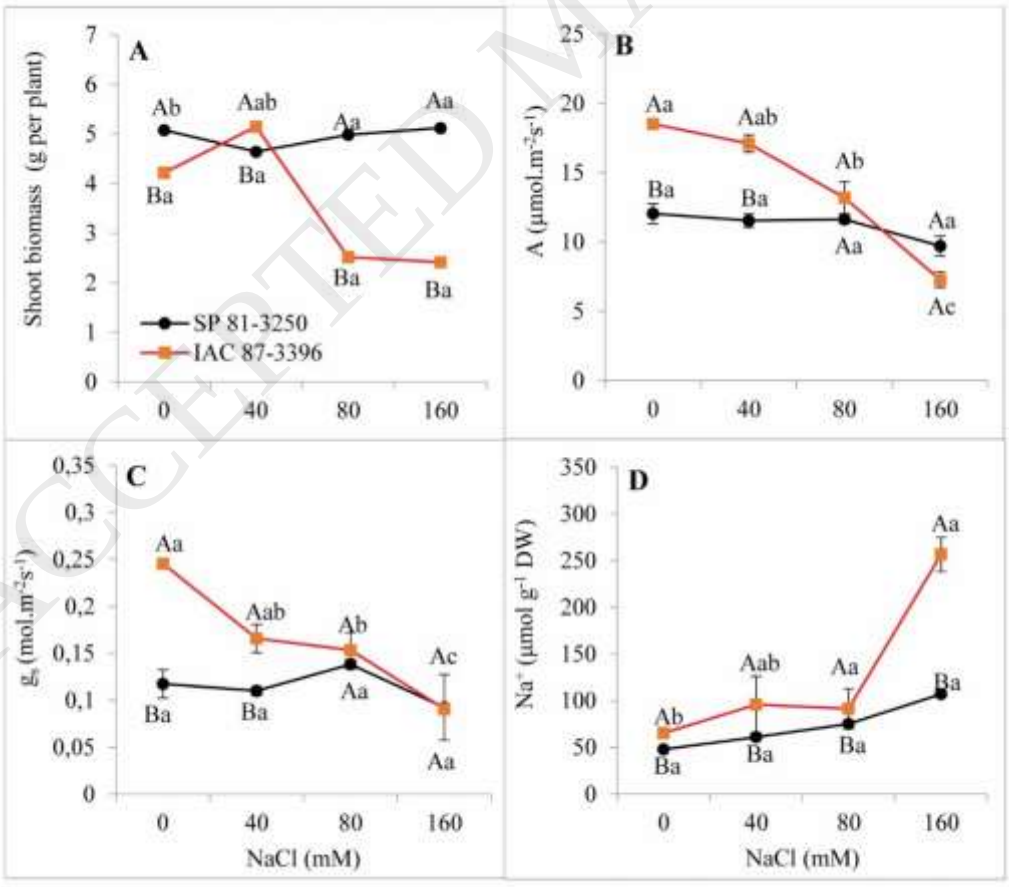
2.8 Statistical analysis

Statistical analysis was performed by the statistical software AgroStat (Barbosa and Maldonado Jr, 2011). The data were submitted to variance analysis by the F test and the significant differences between the treatments were compared by the Tukey test at 5% probability.

3 RESULTS

3.1 Genotype SP 81-3250 is more salt tolerant than IAC 87-3396

The preliminary experiment showed that growth of sugarcane plants for 30 d in saline soil ranging up to 160 mM NaCl had little effect on shoot biomass of SP 81-3250, but the biomass of IAC 87-3396 at 80 mM and 160 mM NaCl was greatly reduced (Figure 2A). Consistent with this, the photosynthetic rate of SP 81-3250 showed no reduction with increasing salinity in contrast to IAC 87-3396 which was reduced at 80 and 160 mM NaCl (Figure 2B). The pattern of stomatal conductance was similar to that for photosynthesis (Figure 2C). This is consistent with the absence of a statistically significant change in C_i (intracellular



CO_2) under salinity (Table 1).

Figure 2. Responses of 60 d old sugarcane plants after growth for 30 d in the NaCl concentrations listed (preliminary experiment). A: Shoot biomass, B: Photosynthesis, C: Stomatal conductance, D: Na^+ concentration. SP 81-3250 is shown with black circles and IAC 87-3396 with red squares. Bars show standard error of mean (n=4). Uppercase letters show statistically significant differences between cultivars by Tukey test at 5% of probability, and lowercase letters show significant differences between NaCl concentrations (within each cultivar) by Tukey test at 5% of probability.

	C_i (intracellular CO_2) (µmol mol^{-1})			
NaCl (mM)	0	40	80	160
SP 81-3250	194 Aa	186 Aa	197 Aa	207 Aa
IAC 87-3396	186 Aa	177 Aa	186 Aa	198 Aa

	E (transpiration) (mmol m^{-2} s^{-1})			
NaCl (mM)	0	40	80	160
SP 81-3250	1.65 Ba	1.66 Ba	1.97 Aa	1.49 Aa
IAC 87-3396	2.35 Aa	2.31 Aa	2.08 Aa	1.48 Aa

Table 1. Intracellular CO_2 and transpiration of 60 d old sugarcane plants after growth for 30 d in the NaCl concentrations listed (preliminary experiment). Photosynthesis and stomatal conductance are shown in Figure 2B and 2C respectively. Values are means of 4 replicates. Uppercase letter between cultivars and lowercase letter between NaCl concentrations show difference by Tukey test at 5% of probability.

Leaf Na^+ concentrations were higher in IAC 87-3396 than SP 81-3250, especially at 160 mM NaCl (Figure 2D).

These observations were followed up in the subsequent main experiment when plants were harvested at both 15 and 30 d after salt treatment. Differences between cultivars in response to salinity were visually apparent at 30 d but not at 15 d (compare Figure 1B and 1C). Ion and metabolite concentrations were measured in roots and shoots, as well as physiological parameters of leaves that would indicate ionic versus osmotic effects on growth.

3.2 Na^+, K^+ and compatible solute concentrations in shoots and roots

The Na^+ concentration in shoots and roots of SP 81-3250 was higher than IAC 87-3396 even at 15 d. Both cultivars showed higher Na^+ levels as the NaCl concentration in the soil increased but IAC 87-3396 was significantly higher than SP 81-3250 (Figure 3A). At 30 d, cv. IAC 87-3396 presented markedly higher Na^+ uptake in shoots than SP 81-3250 (Figure 3B). In roots, Na^+ concentrations in IAC 87-3396 were a little higher than SP at 15 days, but markedly higher at 30 d of treatment. Interestingly, this difference was also present in the "0" mM NaCl treatment which in fact had a low level of Na^+ due to its presence in the soil without added NaCl (Figure 3C, D).

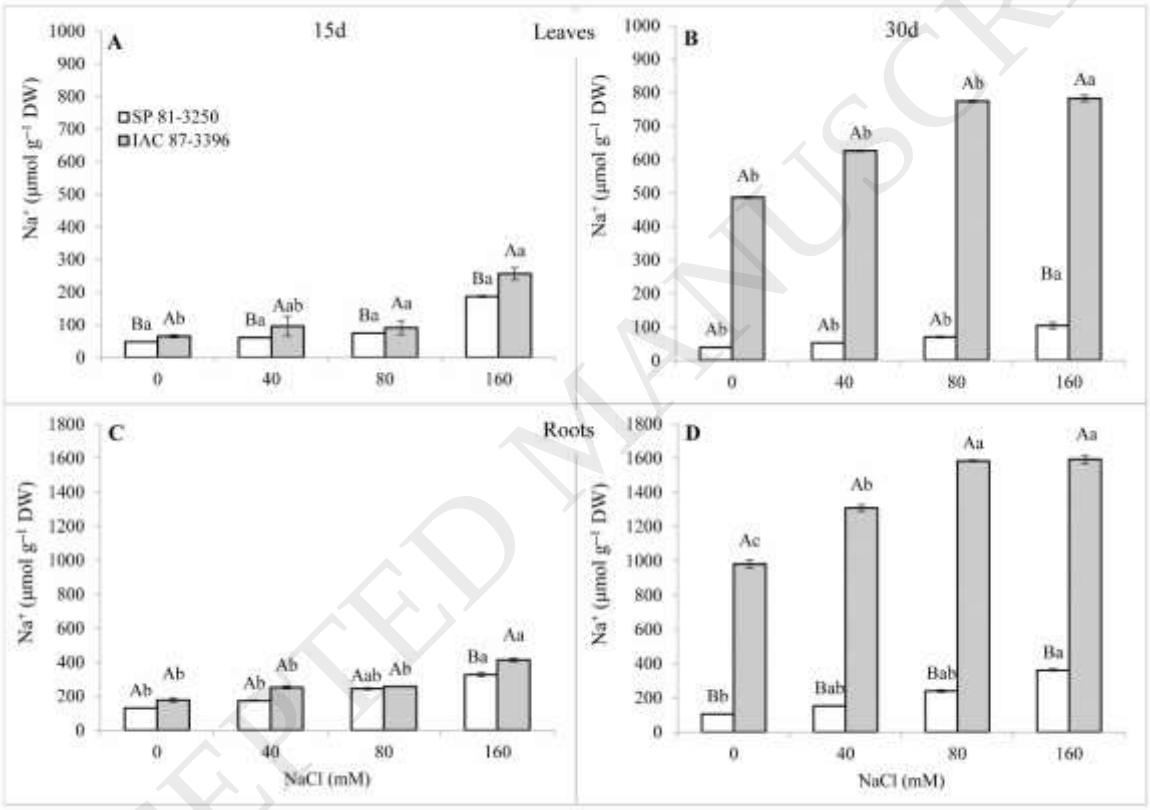


Figure 3. Sodium (Na^+) concentrations in sugarcane plants after growth for 15 or 30 d in NaCl which was added after 30 d of development. A: Leaf Na^+ at 15 d, B: leaf Na^+ at 30 d, C: root Na^+ at 15 d and D: root Na^+ at 30 d. Histograms show SP 81-3250 with open bars and IAC 87-3396 with shaded bars. Bars show standard error of mean (n=4). Uppercase letter between cultivars and lowercase letter between NaCl concentrations show difference by Tukey test at 5% of probability.

The K^+ concentration in leaves was not affected by the salinity treatment, and neither was the K^+ concentrations in roots of either cultivar (Supplemental Figure 1). The Na^+/K^+ ratio therefore was a little lower in SP 81-3250 than IAC at 15 d and much lower at 30 d of treatment for both shoot and roots (Figures 4A to 4D).

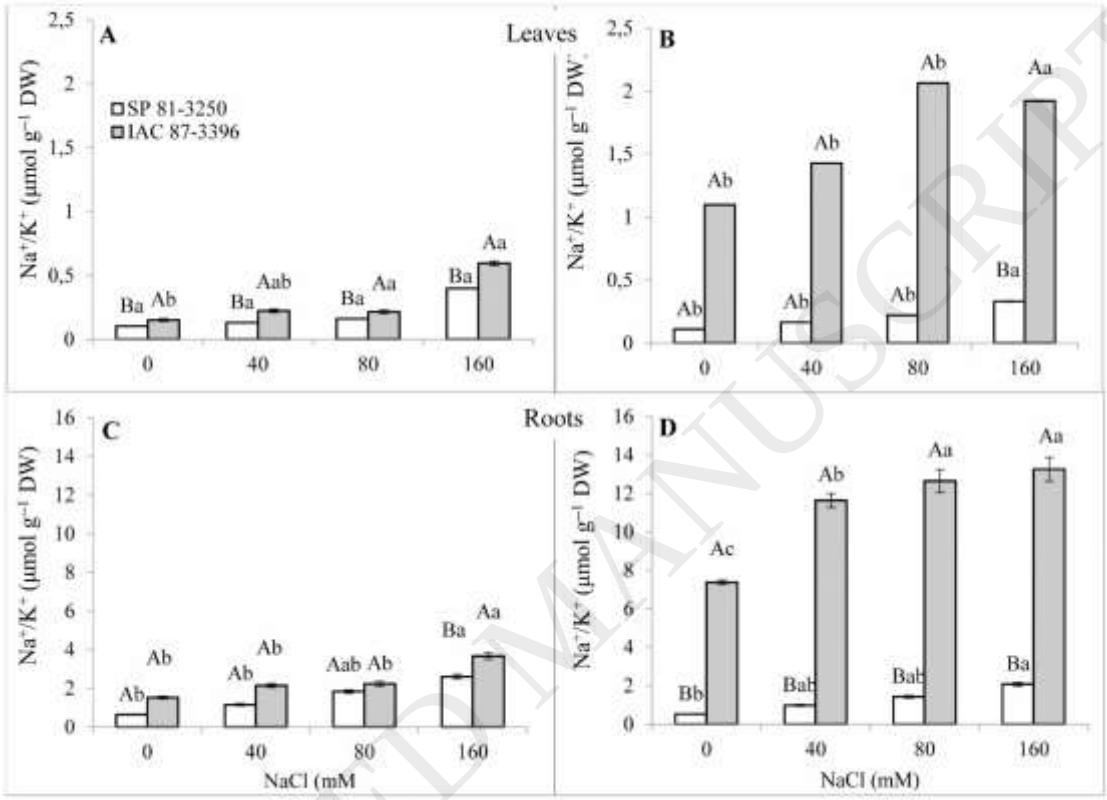


Figure 4. Na^+/K^+ ratio of sugarcane plants after growth for 15 or 30 d in NaCl which was added at 30 d. A: Leaf Na^+/K^+ at 15 d, B: leaf Na^+/K^+ at 30 d, C: root Na^+/K^+ at 15 d and D: root Na^+/K^+ at 30 d. Bars show standard error of mean (n=4). Uppercase letter between cultivars and lowercase letter between NaCl concentrations show difference by Tukey test at 5% of probability.

The **c**ompatible osmolytes proline (Pro) and glycine betaine (GB) were measured in the younger fully expanded leaves and total roots. At 15 d, when submitted to the higher severity of stress (80-160 mM NaCl) the cv. SP 81-3250 showed a higher increase of free proline in leaves than cv. IAC 87-3396, but at 30 d there was no significant difference between cultivars in either leaves or roots (Figure 5A to 5D).

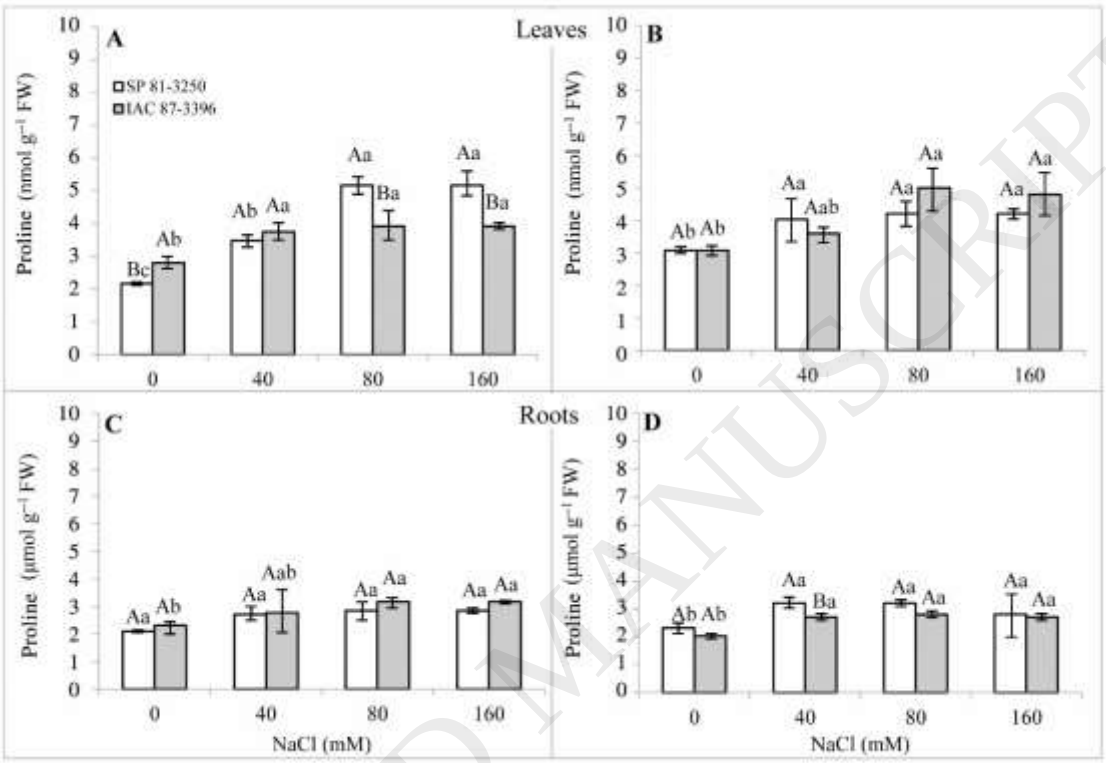


Figure 5. Proline concentration of sugarcane plants after growth for 15 or 30 d in NaCl which was added at 30 d. A: Leaf proline at 15 d, B: leaf proline at 30 d, C: root proline at 15 d and D: root proline at 30 d. Bars show standard error of mean (n=4). Uppercase letter between cultivars and lowercase letter between NaCl concentrations show difference by Tukey test at 5% of probability.

GB concentrations were lower than Pro, and for the salinity treatments were lower at 30 d than 15 d (Figure 6A, B). At 30 day, GB in roots of cv. SP 81-3250 were about twice that of IAC 87-3396 and increased slightly with salinity treatment (Figure 6D).

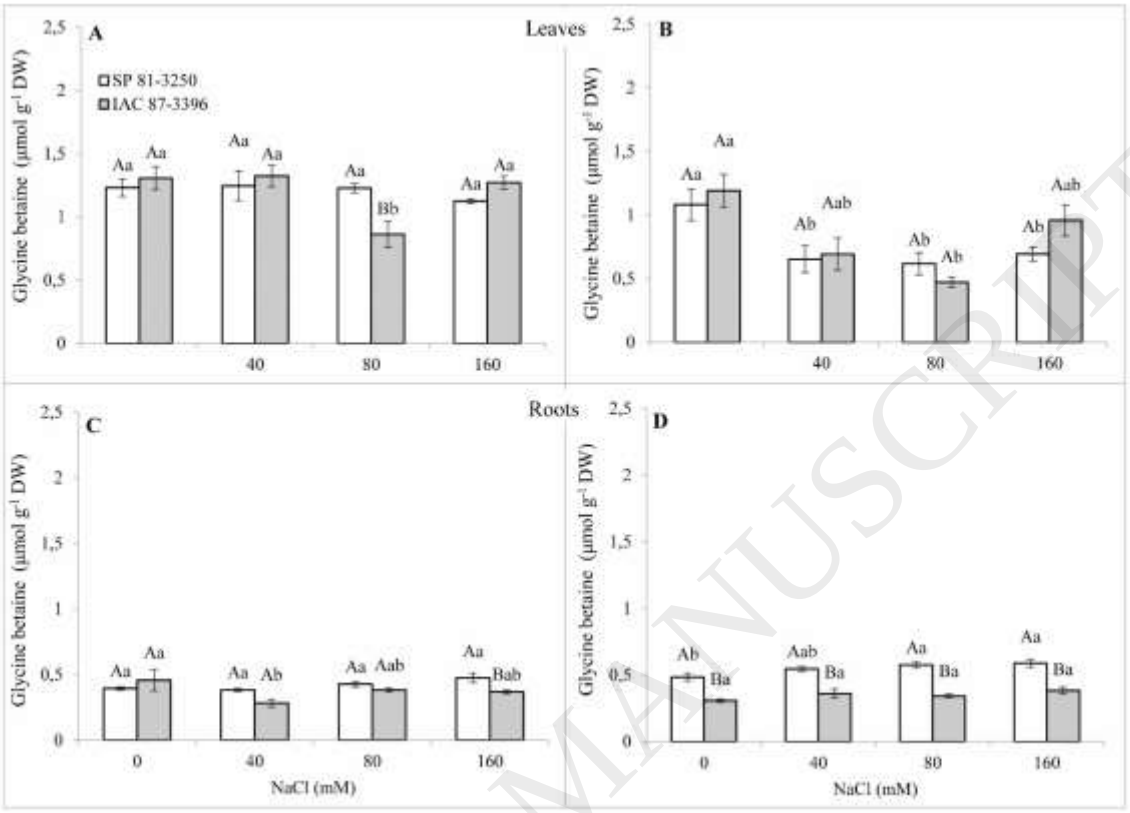


Figure 6. Glycine betaine concentration of sugarcane plants after growth for 15 or 30 d in NaCl which was added at 30 d. A: Leaf glycine betaine at 15 d, B: leaf glycine betaine at 30 d, C: root glycine betaine at 15 d and D: root glycine betaine at 30 d. Bars show standard error of mean (n=4). Uppercase letter between cultivars and lowercase letter between NaCl concentrations show difference by Tukey test at 5% of probability.

Sucrose averaged about 60 and 100 mg g^{-1}DW at 15 and 30 d respectively with no statistically significant different between genotypes or between treatments at either time (Supplemental Figure 2).

3.3 Lipid peroxidation: MDA and H_2O_2

MDA (malondialdehyde) concentrations did not increase at 15 d in either cultivar (Figure 7A), however at 30 days, it increased in cv. IAC 87-3396 under the highest NaCl concentration, 160 mM NaCl (Figure 7B). H_2O_2 concentration in cv. IAC 87-3396 increased at the higher salinity treatments at both 15 and 30 d (Figure 7C, D). H_2O_2 in cv. SP 81-3250 was lower at 30 d than 15 d (Figure 7D).

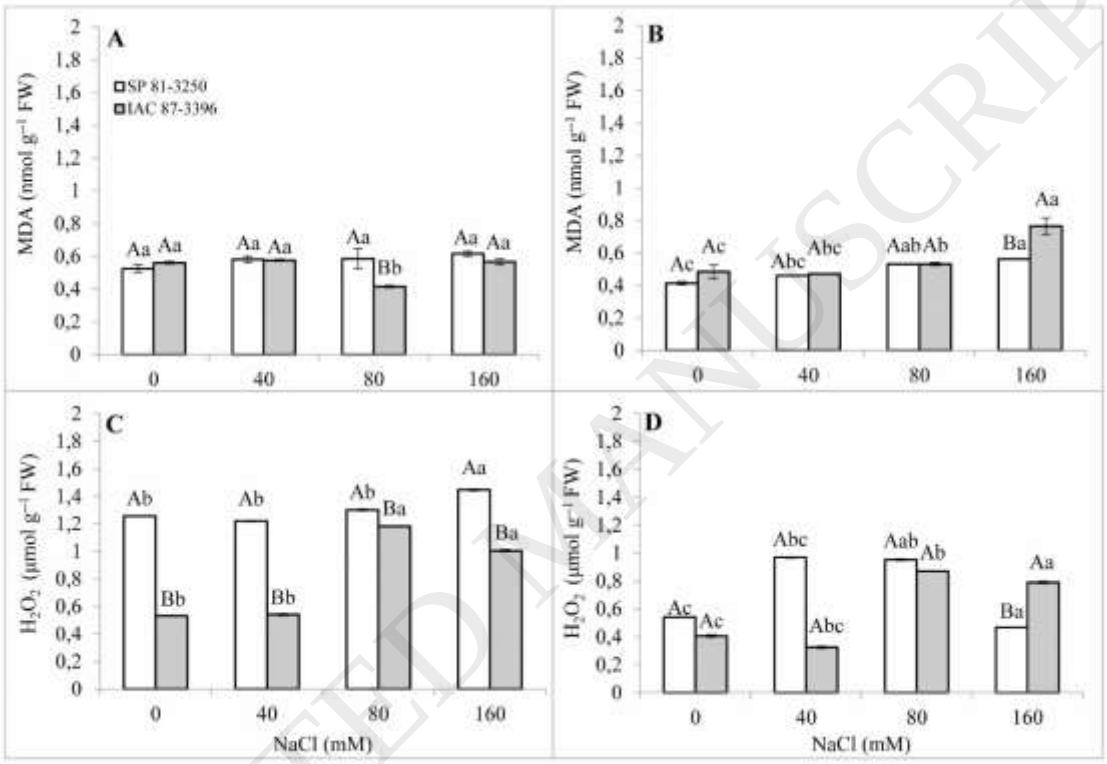


Figure 7. Lipid peroxidation in leaves after growth for 15 or 30 d in NaCl which was added at 30 d. A: MDA at 15 d, B: MDA at 30 d, C: H_2O_2 at 15 d and D: H_2O_2 at 30 d. Bars show standard error of mean (n=4). Uppercase letter between cultivars and lowercase letter between NaCl concentrations show difference by Tukey test at 5% of probability.

3.4 Water relations and photosynthetic capacity

The pre-dawn water potential (Ψ_w) values became more negative with increasing salinity treatment, as would be expected, but there was no statistically significant difference between genotypes or between treatments ($p \geq 0.05$) in both measurement periods (Table 2).

Salinity treatments did not decrease the chlorophyll content of the uppermost leaves in either cultivar (Table 2). It did not affect the quantum yield of photosystem II as shown by the measurement of chlorophyll fluorescence, Fv/Fm (Table 2). Both these results indicate no injury to plants, at least to the leaves that were measured.

Table 2. Total chlorophyll, Fv/Fm and water potential of sugarcane plants after growth for 15 or 30 d in NaCl which was added at 30 d. Values are means of 4 replicates Uppercase letter between cultivars and lowercase letter between NaCl concentrations show difference by Tukey test at 5% of probability.

	Total chlorophyll (SPAD)							
	15 d				30 d			
NaCl (mM)	0	40	80	160	0	40	80	160
SP 81-3250	42.9Aa	42.5Aa	41.3Aa	39.2Aa	43.4Aa	44.3Aa	43.4Aa	38.5Aa
IAC 87-3396	40.1Aa	39.0Aa	38.0Aa	35.0Aa	40.9Aa	39.0Aa	41.0Aa	35.0Aa

	Fv/Fm							
	15d				30d			
NaCl (mM)	0	40	80	160	0	40	80	160
SP 81-3250	0.79Aa	0.78Aa	0.77Aa	0.78Aa	0.77Aa	0.78Aa	0.77Aa	0.77Aa
IAC 87-3396	0.78Aa	0.78Aa	0.79Aa	0.79Aa	0.79Aa	0.80Aa	0.79Aa	0.80Aa

	Water potential (-MPa)							
	15d				30d			
NaCl (mM)	0	40	80	160	0	40	80	160
SP 81-3250	0.17Aa	0.26Aa	0.31Aa	0.32Aa	0.16Aa	0.23Aa	0.26Aa	0.30Aa
IAC 87-3396	0.10Aa	0.21Aa	0.25Aa	0.26Aa	0.09Aa	0.17Aa	0.21Aa	0.31Aa

3.5 Growth

Shoot biomass of cv. SP 81-3250 at 15 d was not affected by salinity, however growth of IAC 87-3396 was reduced in the two higher salinity treatments (Figure 8A). At 30 d there was no statistically significant effect of salinity on SP 81-3250 on shoot biomass but that of IAC 87-3396 was reduced by salinity (Figure 8B). Differences between cultivars at 15 and 30 d are apparent in Figure 1B-1E. Root biomass of IAC 87-3396 was lower than SP 81-3250 in the absence of salinity. There was no effect of salinity on the growth of roots in either cultivar at either 15 or 30 d of treatment (Figures 8C, D).

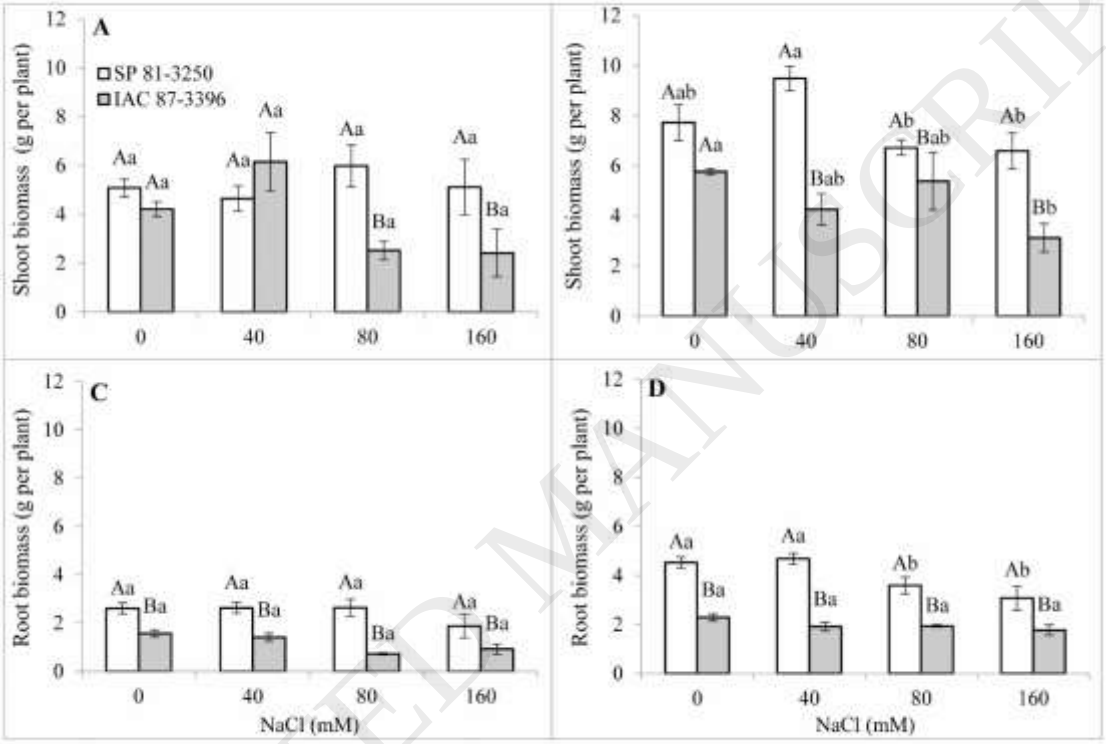


Figure 8. Dry weight of sugarcane plants after growth for 15 or 30 d in NaCl which was added at 30 d. A: Shoot biomass at 15 d, B: Shoot biomass at 30 d, C: Root biomass at 15 d and D: Root biomass at 30 d. Bars show standard error of mean (n=4). Uppercase letter between cultivars and lowercase letter between NaCl concentrations show difference by Tukey test at 5% of probability.

Plant height was not statistically significantly affected by salinity at 15 or 30 d of treatment (Supplemental Figure 3A, B). Leaf area was lower in cv. IAC 87-3396 than cv. SP 81-3250 in the absence of salinity and was drastically reduced by 80 and 160 mM NaCl after 15 d (Supplemental Figure 3C). After 30 d the reduction in leaf area occurred at 40 mM NaCl

as well, while cv. SP 81-3250 was not affected (Supplemental Figure 3D). Root density showed little effect of salinity (Supplemental Figure 3E, F).

4. Discussion

4.1 Different sugarcane genotypes respond differently to salinity: cv. IAC 87-3396 shows greater sensitivity with reduced growth and gas exchange

Photosynthetic response of plants to salinity is complex, since it depends on the salt concentration in the soil as well as the duration of stress, so the symptoms and consequences will vary accordingly (Chaves et al., 2009). The plants of cv. IAC 87-3396 showed a marked reduction in stomatal conductance, foliar area and photosynthesis according to the severity of stress. Stomatal conductance reduction and the consequent lower CO_2 assimilation means the photosynthetic apparatus is exposed to excessive radiation, producing reactive oxygen species (Rishi and Sneha, 2013) which may explain the greater lipid peroxidation that occurred in cv. IAC 87-3396. In low salinity (0 and 40 mM NaCl) the photosynthetic rate of cv. IAC 87-3396 was higher than cv. SP 81-3250. However, in higher salinity concentrations (80 and 160 mM NaCl), the plants of this cultivar presented a marked reduction of photosynthetic rate, unlike cv. SP 81-3250 which maintained similar rates in all treatments. Chlorophyll content and quantum yield of photosystem II (Fv/Fm) were not significantly altered by salinity in either cultivar, showing that gas exchange was a more sensitive indicator of the effects of salinity on photosynthesis than was chlorophyll fluorescence. This was also found for durum wheat grown at 150 mM NaCl (James et al., 2002).

Salinity reduced the shoot dry mass, height, and leaf area of cv. IAC 87-3396 with little or no effect on cv. SP 81-3250, indicating that the lower productivity observed in cv. IAC 87-3396 is due to a combination of the lower photosynthetic area as well as the lower rate of photosynthesis. The effects of salinity are commonly evident in leaf area reduction, significantly impairing net assimilation and plant productivity (Esteves and Suzuki 2009). Significant effects on photosynthesis and biomass were found in sugarcane plantlets grown on MS medium with 200 mM NaCl for 14 d (Cha-Um et al., 2012), and in 120 day-old plants grown in sand culture with 100 mM NaCl for 11 d (Medeiros et al., 2014). Drastic reductions of biomass, photosynthesis and productivity in genotypes of sugarcane plants were found by Vasantha et al. (2010) with effects more pronounced in the later stages of growth. Previous

studies with sugarcane cultivars RB 86-7515 and RB 86-3129 in a range of NaCl concentrations up to 200 mM showed effects on chlorophyll levels and chlorophyll fluorescence, highlighting the damage caused by high salinity in the photosynthetic apparatus and, therefore, in productivity (Willadino et al., 2011). Salinity also reduced the root dry mass, particularly of cv. IAC 87-3396, but did not affect the root density of either cultivar.

~~Our study illustrated the high genetic variability in sugarcane to productivity both with and without salinity. Plants of cv. IAC 87-3396, although they were released as rustic plants and show good development when in non-limiting situations, under high salinity their productivity was drastically reduced and they were more salt sensitive than plants of cv. SP 81-3250.~~

4.2. The cv. IAC 87-3396 had higher Na^+ accumulation in leaves and roots

Leaf and root concentrations of Na^+ were similar in both cultivars at 15 d, but at 30 days, cv. IAC 87-3396 had much higher concentrations than cv. SP 81-3250, about 8 times higher. This is likely to explain its greater sensitivity. In cv IAC 87-3396 at 80 and 160 mM NaCl the leaf Na^+ was about 800 mmol kg^{-1} DW (Figure 3B). The leaf water content of both cultivars was measured as 2.9 g H_2O per g DW, so the value of 800 mmol kg^{-1} DW would be about 275 mM Na^+ on a tissue water basis. This is higher than the value of 250 mM Na^+ considered to be toxic for other monocot species such as durum wheat (James et al., 2002). In contrast, cv. SP 81-3250

Leaf Na^+ exclusion has been confirmed as a mechanism for salt tolerance in many species. Na^+ exclusion from leaves is associated with salt tolerance in most cereal crops including rice, durum wheat, bread wheat, barley and pearl millet, where genotypes with a lower rate of accumulation of Na^+ tend to be the more salt tolerant and produce greater biomass over time (reviewed by Munns, 2011). ~~Not all species conform to this pattern, for example a study with 7 cultivars of maize found variation in Na^+ exclusion that was not associated with salt tolerance (Alberico and Cramer, 1993) however the NaCl treatment there was only 80 mM~~. A lower rate of Na^+ transport to leaves would reduce the risk of Na^+ accumulating in the cytoplasm. For tolerance of a saline soil, Na^+ concentrations in the cytoplasm must be kept below toxic levels, below 30 mM ~~. Na^+ and Cl^- must be partitioned within cells so that concentrations in the cytoplasm are kept low~~ (Munns and Tester, 2008). Mechanisms for keeping cytoplasmic concentrations of Na^+ at this low level are of two main types: those

minimizing the entry of salt into the root and its transport through the plant, and those minimizing the concentration of salt in the cytoplasm by sequestration in vacuoles. This high degree of exclusion of Na^+ is achieved by (i) tightly controlled uptake from the soil, regulated loading of the xylem, and by retrieval of Na^+ from the xylem as it moves in the transpiration stream to the leaves (Munns, 2011). Cv. SP 81-3250 had much lower degree of accumulation of Na^+ in roots as well as leaves, indicating the exclusion of Na^+ from leaves compared to IAC 87-3396 was due to tightly controlled uptake from the soil and therefore the plant as a whole, rather than retrieval of Na^+ from the xylem stream flowing to the shoots.

A higher K^+ or a lower Na^+/K^+ has also been considered as an essential indicator of salt tolerance (Shabala and Pottosin, 2014). K^+ concentrations in plants usually decrease with NaCl treatment (Shabala and Cuin, 2008), however in sugarcane there are exceptions. A decrease in leaf K^+ with increasing NaCl treatment was found with only one of two cultivars by Gandonou et al. (2011) and Medeiros et al. (2014), while Plaut et al. (2000) found a small increase in both cultivars measured. In this study, K^+ accumulation did not decrease with NaCl treatment although it was lower in IAC 87-3396 than SP81-3250 in all treatments and tissues other than the leaves after 30 d when it was a little higher. Because of the marked differences in Na^+ accumulation between genotypes, the Na^+/K^+ ratio in cv. IAC 87-3396 was greatly higher than in SP80-3250 in both leaves and roots, increasing according to salinity. Na^+ and K^+ cations compete for uptake by membrane transporters (Deinlein et al., 2014) and K^+ deficiency relates to productivity as it is involved in important physiological processes, such as stomatal opening (Benito et al., 2014).

Cl^- uptake is rarely measured for plants growing in NaCl as it is considered less toxic than Na^+ (Munns and Tester, 2008). A comparison between various Cl^- salts (50 mM Cl^-) on photosynthesis and sucrose production in sugarcane found little difference between the effects of NaCl and KCl, and that KCl had the greatest effect on quality of cane juice.

4.3. Compatible osmolytes: Proline increases but glycine betaine does not

Salinity causes a decrease in soil osmotic potential and restricts water uptake by the plant. The accumulation of compatible osmolytes, such as proline and glycine betaine, are important to maintain cellular turgor particularly in cytoplasmic compartments (Negrão et al., 2017). Proline increased in the leaves of both cultivars with increases in salinity, and more so in cv. SP 81-3250. At 15 d, young plants of cv. SP 81-3250 showed a significant change of proline 2.3 to 5.6 µmol g^{-1} FW. These leaves had a water content of 2.9 g H_2O g^{-1} DW (see

above), so this change in proline concentrations is equivalent to a change from 3.1 to 7.5 µmol g^{-1} H_2O or 3.1 to 7.5 mM. An increase of Na^+, if compartmentalized in the vacuole, might stimulate more proline to be synthesized to balance the osmotic adjustment in the cytoplasm. Munns (2011) proposed the value of at least 10 mM of accumulation for a substance to be considered as a compatible osmolyte. If we assume that all the proline is in the cytoplasm, and this occupies 10 % of the volume of the cell, then in the example above the proline would have increased from 31 mM to 75 mM, which is a significant osmotic adjustment (over 0.1 MPa).

Glycine betaine did not accumulate in leaves of sugarcane plants after salt treatment. In contrast, there was a 50 % reduction in leaves of cv. IAC 87-3396 after 30 d with less change in cv. SP 81-3250. On the other hand, in roots of stressed plants of cv. SP 81-3250 there was a small accumulation of GB, which coincided with a small but significant increase in Na^+. However, stressed plants of cv. IAC 87-3396 that accumulated much higher Na^+ in the roots did not show increase of GB. GB is considered to benefit plant tolerance to salinity through osmotic adjustment of cells, and even at relatively low levels, around 5 µmol g^{-1} FW, protect cell membranes and the photosynthetic apparatus (Kumari et al., 2015). Our results show a lower concentration of this in sugarcane and do not support a significant effect of GB on either osmotic adjustment or as a compatible metabolite in young plants.

Although sucrose did not show great variation between treatments and genotypes, this carbohydrate can be considered an osmolyte compatible in this work, since high values were found for this compound. Sucrose concentrations for both cultivars were about 60 and 100 mg g^{-1}DW for 15 d and 30 d respectively, which would be about 60 mM and 100 mM respectively on a leaf tissue water basis.

4.4. Proline may have acted as an antioxidant in ROS elimination in young sugarcane plants of cv. SP 81-3250

Compatible osmolytes are also considered to be non-enzymatic antioxidant compounds, so it can be assumed that the oxidative stress caused by salinity in both cultivars may have been ameliorated mainly by a significant proline increase. Some authors report that, in addition to osmoprotection functions, proline also functions in the ROS elimination by non-enzymatic antioxidant activity (Molinari et al., 2007; Dolatabadian et al., 2008, Szabados and Savoure, 2010; Rejeb et al., 2014). Studies with transgenic sugarcane plants transformed with the P5CS gene from proline synthesis have demonstrated that the accumulation of this amino acid acts as an antioxidant, protecting against oxidative stress generated by salinity (Guerzoni et al.,

2014; Iqbal et al., 2014). A higher increase of proline and GB and lower lipid peroxidation in CoM-265 (salt tolerant) sugarcane cultivar, compared to cv. CoC-671 (salt susceptible), was found by Satbhai and Naik (2014), demonstrating the positive effect of compatible osmolytes on plants under salinity. GB did not have that same function in our studies as it was reduced in stressed plants.

Both H_2O_2 and MDA are considered oxidative stress indicators in plants. Lipid peroxidation evaluated by MDA accumulation in plants under salinity confirms the results of higher sensitivity of cv. IAC 87-3396 to salt stress. Higher concentrations of NaCl, 80 and 160 mM, increased the H_2O_2 concentration in IAC at 15 days, although no significant increase in MDA was observed, presumably because peroxide accumulation had not yet altered the membrane lipids. At 30 days, the H_2O_2 accumulation that occurred in stressed plants of cv. IAC 87-3396 was greater than in cv. SP 81-3250 by 35%. This may be related to the smaller increase of Pro in cv. IAC 87-3396 compared to cv. SP 81-3250. In cv. SP 81-3250 the Pro levels were significantly higher at the more severe NaCl concentrations, which may have conferred tolerance to oxidative stress, since this cultivar showed a reduction in the H_2O_2 content at the highest NaCl concentration.

5. Conclusion

The sugarcane cultivars showed great differences in growth response to salinity. It is likely that the higher Na^+ accumulation by cv. IAC 87-3396 caused the greater decrease of productivity. Calculations showed that leaf Na^+ concentrations reached about 275 mM in plants grown at 80 and 160 mM in this cultivar while being only 35 in cv. SP 81-3250.

Genetic variation in salt tolerance is important for sustainable sugarcane production on saline soils as salt in the soil is difficult to remove, and the choice of salt-tolerant plants is the best solution for each specific environment. Our work revealed biomarkers in young sugarcane plants for selection for tolerance of saline conditions. These include the reduction of leaf area and gas exchange with consequent reduction in shoot dry matter as important factors to demonstrate relative sensitivity of cv. IAC 87-3396. The lower Na^+ accumulation and higher proline accumulation of cv. SP 81-3250 indicate mechanisms allowing this cultivar to tolerate salt stress. Measurements of leaf Na^+ concentration would be a feasible selection tool to identify salt-tolerant germplasm in breeding programs.

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