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ARTÍCULO DE INVESTIGACIÓN / RESEARCH ARTICLE

RESPONSE TO *in vitro* SALT STRESS IN SUGARCANE IS CONDITIONED BY CONCENTRATION AND CONDITION OF EXPOSURE TO NaCl

Respuesta *in vitro* al estrés salino en caña de azúcar esta condicionada por la concentración y condición de exposición al NaCl

Manuela Maria CAVALCANTE GRANJA[1], Maria Jaislanny LACERDA E MEDEIROS[2], Marina MEDEIROS DE ANDRADE SILVA[3], Terezinha CAMARA[4], Lilia WILLADINO[4], Claudia ULISSES[4].

[1] Universidade Federal de Viçosa, Campus Viçosa. Avenida Peter Henry Rolfs, s/n. Viçosa, MG, Brazil.

[2]Universidade Federal do Piauí, Campus Universitário Ministro Petrônio Portella. Teresina, Brazil.

[3] Instituto Federal de Rondônia, Campus Guajará-Mirim. Avenida Quinze de Novembro, s/n. Planalto,Guajará, Mirim, Brazil.

[4]Universidade Federal Rural de Pernambuco. Rua Dom Manoel de Medeiros, Dois Irmãos. Recife, PE, Brazil.

For correspondence. manukagranja@gmail.com

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ABSTRACT

Salinity is one of the major environmental stress factors that affect crop productivity, as well as interfering with plant growth and development, resulting in reduced production quality. Given this, we highlight the importance of research in response to plants subjected to salt stress in order to assess the physiological and biochemical behavior of genotypes, with the objective of selecting the more tolerant. One way to ensure the uniformity of the response of the plants is through in vitro cultivation, which allows control of the cultivation conditions. Therefore, the objective of this work was to evaluate the response of two commercial varieties of sugar cane exposed to saline stress in different conditions (gradual and abrupt). Two varieties of sugarcane (RB931011 and RB872552) were subjected to in vitro salt stress by NaCl (56 mM and 112 mM), either gradually or suddenly. The responses of the enzymatic antioxidant system (catalase, peroxidases and ascorbate peroxidase) and free proline, as well as the Na^+ and K^+ contents, were assessed 30 days after the beginning of the treatments. Differences were observed in the responses of the varieties as a function of the induction mode of salt stress, graded or by shock, rather than as a function of the NaCl concentrations in the culture medium. The stress response is therefore conditioned not only by salt concentration, but also by the form of exposure to salt.

Keywords: antioxidant enzymes, *in vitro* culture, oxidative stress, salinity.

RESUMEN

La salinidad es uno de los principales factores de estrés ambiental, además de interferir en el crecimiento de las plantas perjudica directamente la producción agrícola. En ese contexto, se destaca la importancia de investigaciones direccionadas a la respuesta de las plantas sometidas al estrés salino, con el fin de evaluar el comportamiento fisiológico y bioquímico con el objetivo de seleccionar genotipos tolerantes a dicha condición. Una de las técnicas más utilizadas para uniformizar la respuesta de las plantas a una condición en particular es el cultivo de tejidos *in vitro*. Por lo tanto, el objetivo de este estudio fue evaluar la respuesta de dos variedades comerciales de caña de azúcar (RB931011 e RB872552) expuestas a estrés salino con NaCl (56 mM e 112 mM) en diferentes condiciones, gradual y abrupta. Las respuestas del sistema antioxidante enzimático (catalasa, peroxidasa y ascorbato peroxidasa) y prolina libre, asi como las concentraciones de Na^+ e K^+ fueron evaluadas 30 días después del inicio de los tratamientos. Fueron observadas diferencias en la respuesta de las variedades en función del modo de inducción del estrés salino, graduado o abrupto, y no solo en función de las concentraciones de NaCl en el medio de cultivo. La respuesta al estrés es condicionada no solo por la concentración de sal sino también por la forma de exposición al medio salino.

Palabras clave: cultivo *in vitro*, enzimas antioxidantes, estrés oxidativo, salinidad.

INTRODUCTION

Salinity is one of the major environmental stress factors that affect crop productivity (Grewal, 2010), as well as interfering with plant growth and development, resulting in reduced production quality (Basalah, 2010). Salt stress is a phenomenon that occurs due to the high concentration of salts in soils, which are mainly composed by sodium chloride (NaCl). This type of stress can occur in three ways: (1) gradual exposure to increased NaCl concentration (Joseph *et al*., 2015), or (2) the plant sudden exposure to high levels of NaCl (Wegner *et al*., 2011), or (3) the combination of both. Salt shock, even though is the stress most commonly applied in scientific experiments, rarely occurs in natural conditions, as the increase of NaCl occurs gradually in the environment (Shavrukov, 2013). Sudden salt stress immediately induces an osmotic shock when plants are rapidly exposed to large osmolality differences with a high NaCl concentration and this is what may occur in agriculture when saline water is applied to irrigation (Wegner *et al*., 2011; Shavrukov, 2013).

The sugarcane (*Saccharum* sp.) is part of the Poaceae and it is considered to be moderately sensitive to salinity. Also, this plant presents a yield decrease of up to 50 % in soils with electrical conductivity of 10.4 dS m^{-1} (Santana *et al*., 2006). Although traditionally cultivated in the wetlands of Mata and Litoral in the northeastern region of Brazil, sugarcane crop has been expanding to semi-arid regions. Studies on the tolerance of sugarcane to salinity are becoming increasingly frequent due to the interest of using this crop for the production of bioenergy and other by-products (Patade *et al*., 2012; Melo *et al*., 2014). The development and selection of genotypes involves studies on the tolerance mechanisms of the culture, and, therefore, it is necessary to know plants' physiological responses to salt stress. In vitro tissue culture techniques to study the plant's physiology and biochemistry under stress conditions are important tools because they enable control over the culture's conditions and its homogeneity (Murshed *et al*., 2015). Two sugarcane genotypes developed for the Northeast region of Brazil by the Sugarcane Genetic Improvement Program of the Interuniversity Network for the Development of the Sugarcane Sector (RIDESA) were assessed and evaluated to the gradual and sudden addition of NaCl to the culture medium during the plant's in vitro development. Due to the scarcity of information available about the effects of the application of graded saline stress has on the antioxidative defense system of in vitro sugarcane plants, biochemical aspects were evaluated in the two sugarcane varieties with the aim to investigate the genotypes' abilities to maintain ionic and redox homeostasis depending on the form of stress application.

MATERIALS AND METHODS

Two commercial sugarcane genotypes (RB931011 and RB872552) were submitted to salt stress in vitro by addition of NaCl to the culture medium. The plants were obtained from the in vitro growth in a Temporary Immersion System, provided by the Biofábrica Governador Miguel Arraes, from the Northeast's Center of Strategic Technologies (CETENE). During the experiment's initial stage – acclimatization- the culture was maintained in basic liquid medium (BLM), consisting of MS (Murashige and Skoog, 1962) minerals and vitamins plus sucrose (3 %). The pH of the medium was adjusted to 5.8 before autoclaving at 120 °C and 104 kPa for 20 minutes. A total amount of 20 mL of medium was dispensed into 250 mL capacity pots. A sum of 1,200 plants were selected, 600 plants of each variety, during the final phase of rooting, done in temporary immersion bioreactors, they were submitted to one month acclimatization before the treatment application. After this period of 30 days of acclimation, five plants with greater vigor were selected and transferred to each flask with approximately 20 mL of medium, in which the different salt concentrations were applied. The experiment was maintained for 35 days in the plant growth chamber at a temperature of 27 ± 2 °C with a photoperiod of 16 hours, light intensity of 50 $\mu mol.m^{-2}\ s^{-1}$ provided by cold white light. Two salt treatments were established (T1 = 56 mM NaCl, T2 = 112 mM NaCl), in addition to a control treatment (T0) without the addition of NaCl. The graded treatments (T1g and T2g) were applied by transferring the five plants to pots containing the following NaCl levels: T1g = 8 mM NaCl; T2g = 16 mM NaCl. From these concentrations (8 and 16 mM NaCl), five milliliters (5 mL) of medium were added every five days, increasing the salt concentration in 8 mM or 16 mM NaCl for the treatments T1g and T2g, respectively, until each treatment reached its final concentration, which were 56 mM for T1g and 112 mM for T2g, as shown in Table 1. For the salt shock treatment, the plants were transferred directly to culture medium with the concentrations of 56 mM (T1c) and 112 mM NaCl (T2c).

The experimental delimitation was completely randomized in factorial scheme 3x2x2, corresponding to NaCl concentrations (0, 56, 112 mM), stress condition (gradient or shock) and both varieties. Twenty replicates were maintained per treatment and the experimental unit was composed of a flask with five plants. The plants of each treatment were assessed for their antioxidative response by analyzing the enzymatic activities of ascorbate peroxidase (APX), catalase (CAT) and peroxidases (POD), determining the contents of proline, sodium (Na^+) and potassium (K^+) and through the calculation of Na^+/K^+ ratio. Of the 20 replicates for each treatment, 15 were used to determine proline and Na^+ and K^+ contents and the others were used for enzymatic and total soluble protein analyzes. The collection of fresh and dry matter for these analysis was performed at 35 days. The protein extraction for the enzymatic analysis followed the methodology developed by Azevedo *et al*. (1998). The enzymatic extracts were obtained from the maceration of 0.2

Table 1. NaCl increase during the establishment of salt stress by gradual induction treatments in *in vitro* sugarcane varieties throughout the experimental period.

Treatments / NaCl final concentration (mM)		Days to achieve the final NaCl level							
		0	5	10	15	20	25	30	35
		Addition of NaCl (mM)							
T0g	0	0	0	0	0	0	0	0	Collect
T1g	56	0	8	16	24	40	48	56	Collect
T2g	112	0	16	32	48	80	96	112	Collect

g of leaves previously frozen in liquid nitrogen and stored in a freezer at -20 °C until the start of the extract preparation. All enzymatic analysis were performed by spectrophotometry and the steps were performed in the cold (in an ice bath). Protein quantification followed the methodology described by Bradford (1976). The following methodologies were used to determine the enzymatic activity: catalase (EC 1.11.1.6) (Beers and Sizer, 1952), peroxidase (EC 1.11.1.7) (Fatibello-Filho and Vieria, 2002), ascorbate peroxidase (EC 1.11.1.1) (Nakano and Asada, 1981). Free proline analysis was done using the methodology developed by Bates (1973).

The Na^+ and K^+ contents in the plant's aerial part were determined after performing nitric-perchloric acid digestion of the dry material by flame spectrophotometry (Malavolta, 1989). The quantitative data were submitted to analysis of variance and the means were compared by Tukey test, at a 5 % probability level, using the program ASSISTAT.

RESULTS

CAT activity in plants treated with 112 mM exceeded that of the other treatments, regardless of the variety. The effect of the interaction between varieties and the kind of stress implementation was significant (Fig. 1A). CAT activity did not differ between varieties when stress was applied gradually, however, when it was induced suddenly, the variety RB 872552 presented higher CAT activity in relation to the graded stress and exceeded the variety RB 931011 in this aspect. In both varieties, salt treatments caused a decrease in POD activity in relation to the control, but there was no difference between treatments with 56 and 112 mM NaCl. Regarding the stress implementation, it was observed that the activity of POD in the variety RB931011 was higher when stress was gradually imposed and exceeded that of the variety RB872552. When NaCl was added to the culture medium suddenly, the activity of POD did not differ among the varieties. No effect of the stress inducing form was observed on the variety RB872552 (Fig. 1B). The condition of stress induction allowed to distinguish a greater defensive efficiency, especially in the variety RB931011. The evaluation of the interaction between different varieties and amount of NaCl showed that the APX of the variety RB931011 did not change due to the presence of NaCl. On the other hand, in the

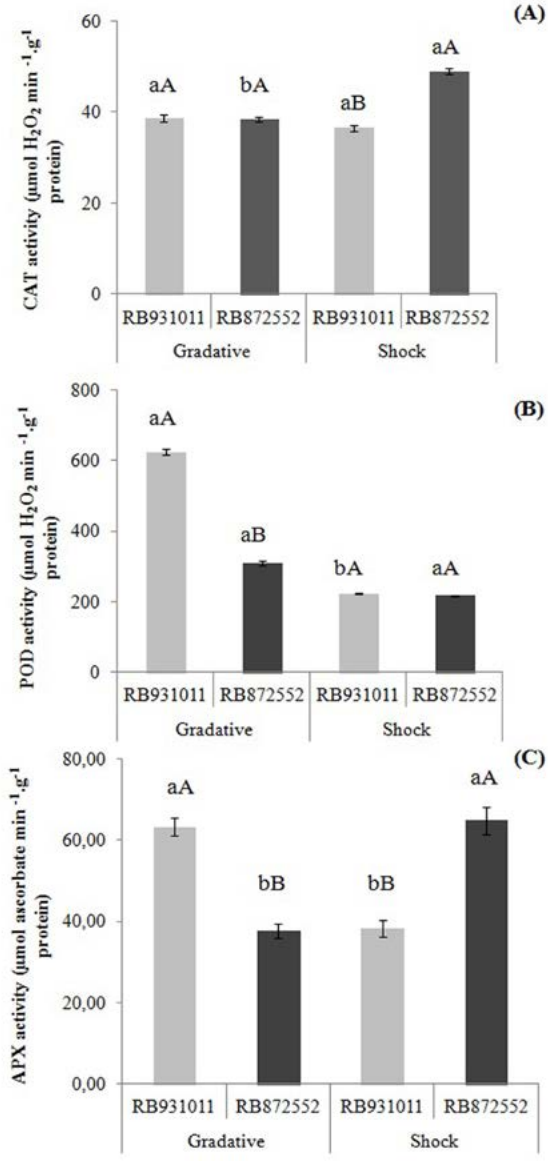

Figure 1. Activity of CAT (A), POD (B) and APX (C) enzymes in the sugarcane varieties RB 931011 and RB 872552 grown *in vitro* and submitted to gradual or sudden NaCl application to the culture medium. Identical letters, upper case between varieties and lower case letters between conditions of stress application, do not differ among themselves by the Tukey test at 5 % probability.

variety RB872552 this enzymatic activity was higher in plants submitted to 112 mM NaCl than in those of other treatments (data not shown). However, the effect of the interaction between the varieties and the salt stress induction condition was significant (Fig. 1C). In to the variety RB931011, the APX activity was higher through a gradual increase in NaCl concentration. The variety RB872552, however, presented the highest enzymatic activity when submitted to osmotic shock than when treated with a gradual increase of the salt concentration in its culture medium. On the other hand, in the variety RB931011, the APX activity was lower than in RB872552 and did not vary due to the NaCl presence in the culture medium.

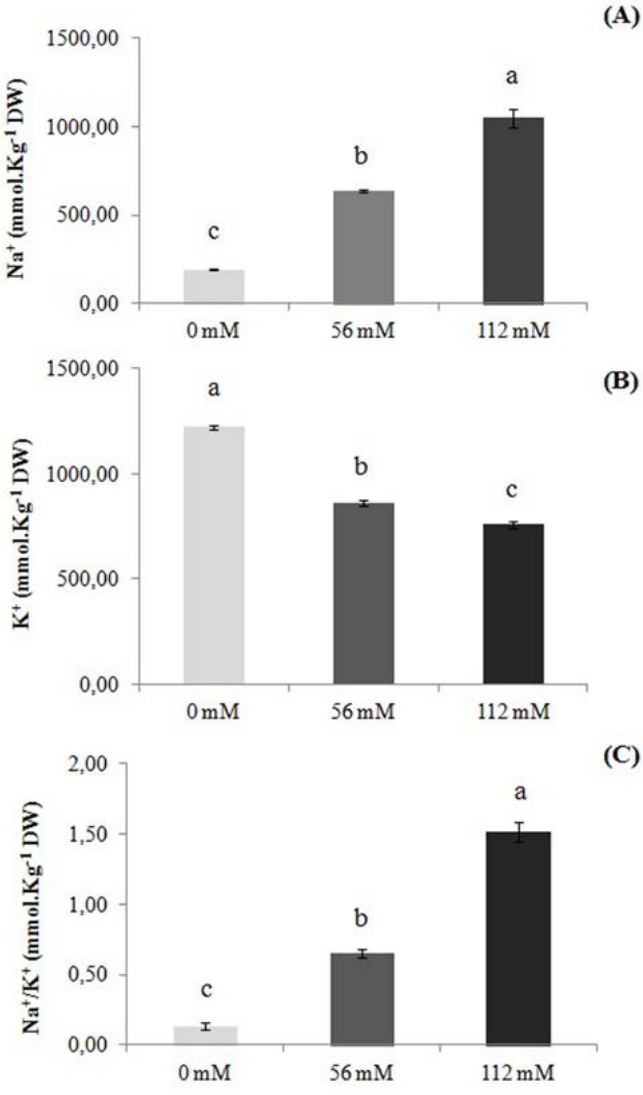

Figure 2. Concentration of Na^+ and K^+ ions and Na^+/K^+ ratio in two sugarcane varieties (RB931011 and RB872552) grown *in vitro* under different levels of NaCl. Different letters over the columns indicate a significant difference by the Tukey test at 5 %. **(A)** Na^+ content; **(B)** K^+ content and **(C)** Na^+/K^+ ratio.

Potassium (K^+) and sodium (Na^+) content, as well as Na^+/K^+ ratio, presented the following aspects: There was a positive association between the NaCl concentration in the culture medium and the sodium (Na^+) content, displaying an accumulation in the aerial part of the varieties analyzed. Fig. 2 shows an increase (7.8 times) in Na+ content and a decrease (1.7 fold) in K^+, with a consequent increase in the Na+/K+ ratio caused by the addition of NaCl in the in vitro culture medium. The effect of interaction between NaCl amount and the stress inducing condition was significant for Na^+ and K^+ content and Na^+/K^+ ratio. In the non-gradual induction of salt stress, the Na+ content and the Na^+/K^+ ratio were higher than that of the plants subjected to gradient stress (Fig. 3B).

On the other hand, the K+ concentration was higher in the RB872552 variety than in the RB931011 variety. The addition of NaCl caused the accumulation of Na^+ in relation to the control group. The accumulation of this cation was higher in plants in which the addition of NaCl was done at once (Fig. 4A). Plants treated with 112 mm of NaCl gradually applied had a K^+ content 1.6 fold lower compared to the control plants, but this concentration was higher than in plants that were submitted to the same saline

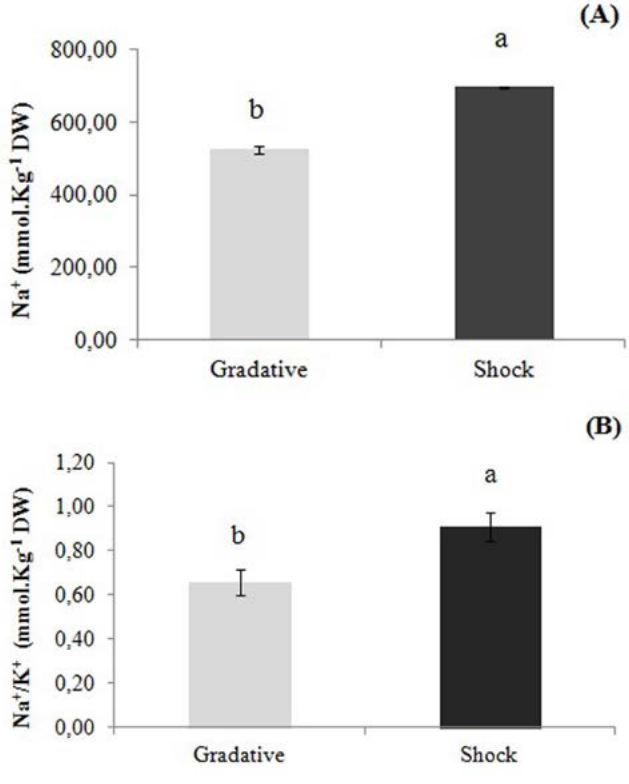

Figure 3. Na^+ content and Na^+/K^+ ratio in two varieties of sugarcane (RB931011 and RB872552) grown *in vitro* under different forms of saline stress induction. Different letters over the columns indicate statistical difference at a 5 % probability level by the Tukey test. (A) Na^+ content and (B) Na^+/K^+ ratio.

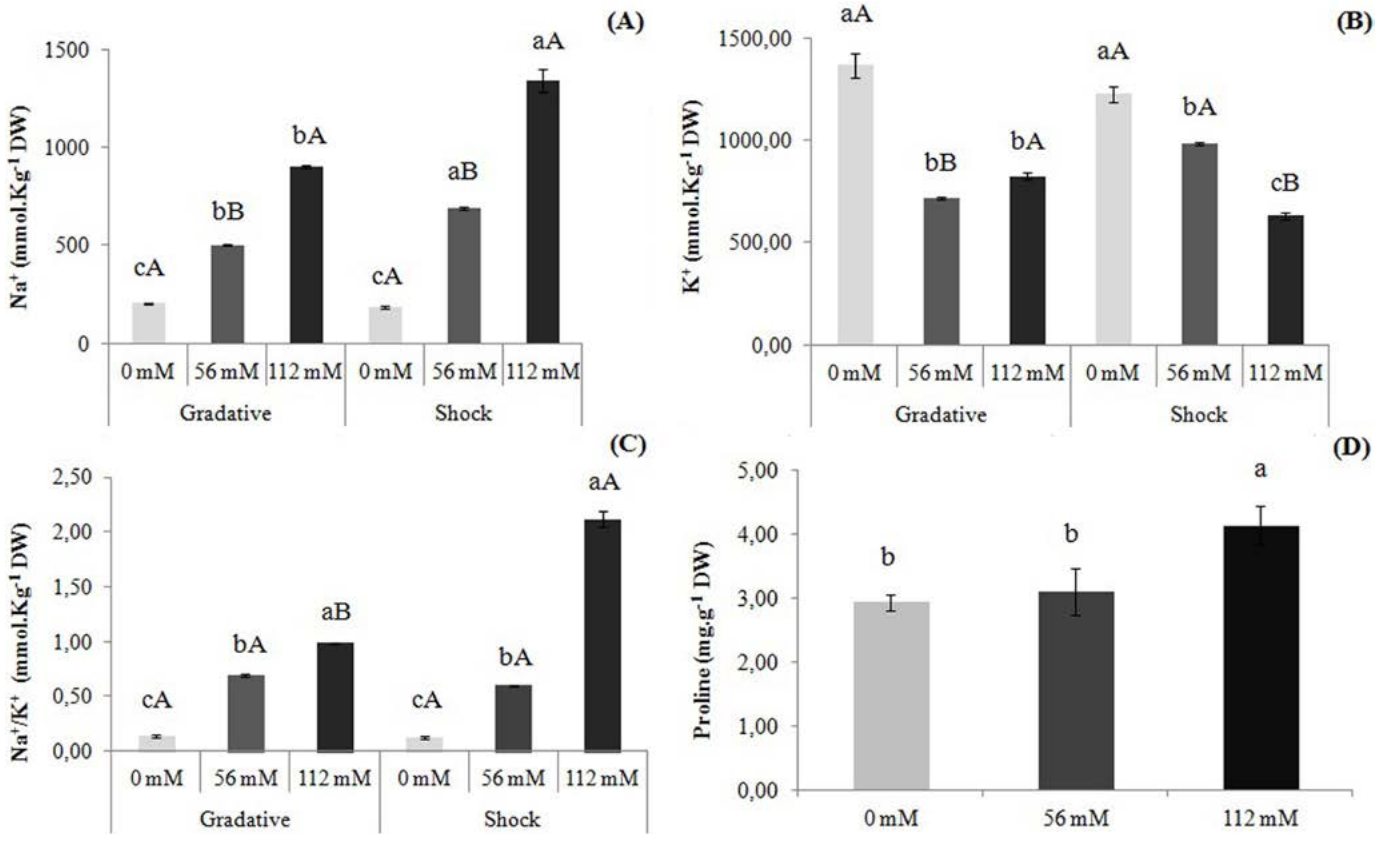

Figure 4. (A) Na^+ content; (B) K^+ content; and (C) Na^+/K^+ ratio in two sugarcane varieties (RB931011 and RB872552) grown *in vitro* in response to the concentration of NaCl (T0 = 0 mM, T1 = 50 mM and T2 = 100 mM) and condition of application (gradual or shock) of salt stress. Identical upper case letters between the conditions of salt stress induction and lowercase between NaCl amount do not differ among themselves by the Tukey test at 5 % probability; (D) Proline content in two sugarcane varieties (RB931011 and RB872552) grown in vitro under different levels of NaCl. Different letters over the columns indicate a significant difference by the Tukey test at 5 %.

concentration (Fig. 4B). The changes in Na^+ and K^+ contents in the tissues of the plants submitted to a sudden increase in the NaCl concentration in their culture medium resulted in a nine fold increase in Na^+/K^+ ratio, whereas in the non-gradual condition this increase was 17.8 higher than the control (Fig. 4C). In the RB872552 variety, the Na^+/K^+ ratio presented a higher value than in the RB931011 variety, with variation starting from the treatment with 56mM NaCl (data not shown).

The proline content increased only in plants submitted to treatment with 112mM of NaCl (Fig. 4D), granting to the sugarcane varieties a greater protection against salt stress. However, this osmolyte did not vary in relation to the form of stress applied or between varieties.

DISCUSSION

The salinity is one of the major abiotic stresses that cause decrease in production and crop yield (Patade *et al*., 2011; James *et al*., 2012; Plazek *et al*., 2013; Munns and Gilliham, 2015). This, because the salinity of soils, causing, directly or indirectly, physiological imbalances to plants (Munns, 2002). These imbalances reach most cultures for being sensitive to high concentrations of salts in the soil (Hasanuzzaman *et al*., 2014). Salt stress, like other environmental stresses, causes an imbalance in the appropriation of reactive oxygen species (ROS), what causes the disorderly increase of these species in the tissues of plants and can lead to severe damage to the plant (Mittler, 2002). As a consequence, the activity of one or more enzymes from the antioxidative system is increased due to the salinity supplementation, which has been recorded for APX enzymes (Maia et al., 2010), CAT and POD (Abogadallah *et al*., 2010; Willadino *et al*., 2011).

Catalase is a very efficient enzyme and it is among the most important ones in the regulation of intracellular H_2O_2 levels in front of abiotic stresses (Mhamdi *et al*., 2010). The high presence of CAT activity in C_4 plants reflects the level of stress to which these plants are subjected (Cuypers *et al*., 2011). This attributes to the enzyme an indispensable function in the process of cellular detoxification from excessive H_2O_2 during a stress situation (Gill and Tuteja, 2010; Cuypers *et al*., 2011). Stress severity can be determined by both its intensity and duration. The abrupt increase in NaCl concentration may

have caused a sudden increase in ROS content and signaled the activation of CAT to ensure the detoxification of H_2O_2 in the variety RB872552. The increase of the CAT activity supposes a possible adaptation to overcome the damages caused by the H_2O_2 accumulation, which is produced during the metabolism and is observed in more tolerant plants (Ackay *et al*., 2010, Willadino *et al*., 2011).

In plants, the POD enzyme composes an antioxidative protection, although in its hydroxyl cycle there is generation of ROS (Bor *et al*., 2003). Willadino *et al*. (2011) and Medeiros *et al*. (2015) observed in different sugar cane varieties a constant behavior of the enzyme up to 100 mM of NaCl, after that concentration there was an increase of the enzyme's activity, which was not observed in the present study, this may have occurred due to differences in tolerance between the varieties used. APX is another enzyme that actively participates in the removal of H_2O_2 by transforming the hydrogen peroxide into water through the oxidation of ascorbate (Teixeira *et al*., 2004). Usually, a high increase in APX activity distinguishes varieties with higher tolerance to NaCl, as it has been observed in *Sesamum indicum* (Koca *et al*., 2007). Activation of APX was also much higher in salt-tolerant wheat cultivars (Mandhania *et al*., 2006). Salinity causes several damage to plants and one of the factors involved for this to happen is the absorption of Na^+. The saline stress leads to the increase of this element with the consequent decrease of K^+ in the plant, both on the leaves and in the roots (Maggio *et al*., 2007). K^+ is the most abundant inorganic cation in plant cells, which composed with nitrogen (N) and phosphorus (P) is fundamental for crop yield (Dreyer, 2014).

The highest Na^+ values in the shock condition, in both varieties, suggests that the sudden increase in salt concentration in the culture medium favors the influx of sodium through the roots, resulting in a greater concentration of sodium in the aerial part and a greater toxicity to the plant. In wheat, the sudden increase in NaCl concentration caused a rise in Na+ content and a reduction in plant growth than when compared to the induction by gradual stress (Almansouri *et al*., 1999). The effect on K^+ content was also more intense in the induction done by osmotic shock. In this case, the K^+ content decreased when salinity increased in the culture medium. When NaCl was gradually added to the culture medium, the cation concentration in the tissue of the salt-treated plants was lower than that of the non-stressed plants. However, K^+ content did not differ between the saline treatments (56 and 112 mM NaCl). The ion selectivity is related to the plants sensitivity to salinity (Willadino *et al*., 1999; Azevedo Neto and Tabosa, 2000). The gradual induction of stress was not as harmful to the uptake of K^+ by the roots as the induction of osmotic shock stress. By adding 112 mM of NaCl suddenly corresponds to a turgor pressure in the order of 0.5 MPa. This pressure is able to plasmolyze the cells in contact with the salt solution. Plasmolysis occurs when the osmotic pressure of the surrounding medium is higher than that of the cells, whose turgor is in the order of 0.4 MPa.

Almodares *et al*. (2014) consider, among other parameters, the relation Na^+/K^+ good indicative of salt tolerance since tolerant plants of sorghum in relation to salinity present a higher relation than the sensitive plants. Therefore, it can be used as an index for sodium toxicity, due to the fact that this ion inhibits the activity of enzymes that require potassium (Greenway and Munns, 1980). The study's data showed an increase in the relationship between sugarcane leaves and the increase in salinity. Garcia *et al*. (2007) and Azevedo Neto and Tabosa (2000) presented similar results with maize leaves. The most significant increase in the non-graded induction results from a higher concentration of Na^+ ions in the culture medium. This ion can then be greatly absorbed by the plant due to the osmotic shock caused. The increase in the Na^+/K^+ ratio indicates an increase in sodium absorption, hindering the entry of other nutrients, which causes an ionic imbalance in the plants (Cusido *et al*., 1987). This imbalance in ionic absorption is probably due to the loss of membrane integrity from the accumulation of Na^+ in the roots (Cramer *et al*., 1985).

Since the K^+ is a major cellular component related to the osmotic balance of the cell and the salt stress affect directly this balance, a way to meet this demand is the osmolytes production supported by plants, such as proline (Shabala and Pottosin, 2014), which actively respond to induce mitigation of the adverse effects caused by osmotic stress (Hasanuzzaman *et al*., 2013; Miri and Mohammad, 2013), as for example the prevention of free radical production or capturing of ROS (Harir and Mittler, 2009). The outcome of increased proline concentration on saline stress has been extensively studied in plants, such as sorghum (De Lacerda *et al.,* 2003), rice (Lima *et al*., 2004) and canola (Saadia *et al*., 2012). Specifically on sugar cane, when subjected to 100 mM of NaCl, it is observed the accumulation of this osmolyte in leaves and roots (García and Medina, 2003; Vasantha and Rajlakshmi, 2009; Karpe *et al*., 2012; Medeiros *et al*., 2015) which corroborates the present work. This accumulation is observed in salt tolerant sugarcane cultivars, because this is a mechanism of protection against salt stress.

The maintenance of redox homeostasis is essential to avoid the oxidative stress from taking place due to the disproportion between formation and appropriation of reactive oxygen species. In order to obtain homeostasis, the plant can use metabolites and enzymes, as discussed in this paper. The duration and intensity of the action of a stressor as well as the association of more than one factor define the severity of stress and define the reaction of the plants.

In this work, it was possible to observe different responses in each of the varieties due to the salt stress induction condition, graded or by shock, rather than due to the NaCl concentrations in the culture medium.

CONCLUSIONS

The varieties differed from each other as to the in vitro salt stress induction. The pattern of a genotype's response to salt stress is conditioned not only by saline concentration, but by the condition of exposure to NaCl–being it graded or by osmotic shock.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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