African Journal of Biotechnology Vol. 11(37), pp. 9028-9035, 8 May, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2906
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Differential responses to salinity stress of two varieties (CoC 671 and Co 86032) of sugarcane (*Saccharum Officinarum* L.)

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Accepted 16 April, 2012

A comparative study was made to assess salt stress responses of sugarcane (*Saccharum officinarum L.*) var. CoC 671 and Co 86032 using *in vitro* plantlets by subjecting them to increasing concentrations of NaCl (0, 50, 100, 150, 200 and 250 mM) and checking relative growth **rate (RGR), membrane damage rate (MDR), soluble proteins, osmolytes (proline, glycine betaine), ions (Na^+ and K^+) and activity antioxidant enzymes (peroxidase, ascorbate peroxidase, guaiacol peroxidase, catalase and superoxide dismutase). As the concentration of NaCl increased, the RGR was found to decrease by 42.1 and 77.7%, the MDA level increased by 32.5 and 55.8% and proline increase of about 43 and 189% was seen in CoC 671 and Co 86032 respectively. CoC 671 was adapted to a higher Na^+ concentration (150 mM) than Co 86032. As for the K^+ accumulation, it displayed similar patterns as in Na^+ accumulation. In general, it was observed that in all cases except catalase, CoC 671 displayed higher tolerance to NaCl (up to 150 mM) than Co 86032 (up to 100 mM). Based on the results, it is suggested that CoC 671 displayed NaCl tolerance up to about 150 mM, while that of Co 86032 was around 100 mM. The study also indicates that *in vitro* plantlets can be used for screening salt tolerance in sugarcane.**

Key words: *In vitro* selection, NaCl salinity stress, relative growth rate (RGR), osmolytes, antioxidant enzymes, sugarcane.

INTRODUCTION

Abiotic stresses like salt and drought induce changes in morphological, physiological, metabolic and molecular attributes that adversely affect plant growth and productivity (Mahajan and Tuteja, 2005). Sugarcane is a typical glycophyte exhibiting stunted growth or no growth under salinity, with its yield falling to 50% or even more of its true potential (Subbarao and Shaw, 1985) which could possibly be due to the accumulation of toxic ions. Being highly crossbred, sugarcane exhibits a significant genetic variability in nature (Wahid et al., 1997). Assessment of the available germplasm against salt stress should become valuable resource for its successful cultivation in problem soils. Salinity in the root zone of sugarcane decreases sucrose yield, through its effect on both biomass and juice quality (Lingle and Wiegand, 1996). Due to this, losses occur in growth rate and sugar content of the plant (Rozeff, 1995). The plants growing under salt stress or water deficit conditions have been investigated in many plants such as rice (Chaum et al., 2007; Castillo et al., 2007) and sugarcane (Patade et al., 2008, 2009; Gondonou et al., 2006; Pagariya et al., 2011).

Plant responses to salt stress are complex involving many genetic networks and metabolic processes and these depend on the inherent salt tolerance of the plant,

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concentration of salt and the duration of exposure (Hasegawa et al., 2000; Munns and Tester, 2008). Plant adaptations to salinity are of three distinct types: osmotic stress tolerance; Na^+ exclusion; and tissue tolerance, that is, tolerance of tissue to accumulated Na^+, and possibly Cl^- (Munns and Tester, 2008). Additionally, osmolytes (betaines and proline) and antioxidant systems (peroxidases like ascorbate peroxidase, guaiacol peroxidase, catalase, and superoxide dismutase) are also important (Greenway and Munns, 1980; Hasegawa et al., 2000; Ashraf and Harris, 2004; Patade and Suprasanna, 2009). In crop improvement programs, it is often desirable to have a good and reliable method for screening large plant population to isolate salt tolerant clone or mutant (in case of mutagenized population). In this regard, we investigated the use of *in vitro* plantlets of two sugarcane cultivars (CoC 671 and Co 86032) to study their differential physiological changes and growth parameters.

MATERIALS AND METHODS

Plant material

Shoot tips of about 5 to 6 cm were excised from the disease free field grown plants of two commercially popular varieties of sugarcane (CoC 671 and Co 86032). Explants were washed in tap water then sterile distilled water and surface sterilized with 0.1% (w/v) $HgCl_2$ solution for 5 to 10 min, and rinsed thrice with sterile distilled water in aseptic conditions. The apical meristems of 4 to 5 month old plants were excised and cultured in liquid MS medium (Murashige and Skoog, 1962) supplemented with 0.1 mg/l kinetin and 0.2 mg/l BAP for 30 to 45 days. The shoots were aseptically excised and transferred in the medium containing 0.1 mg/l kinetin, 0.1 mg/l BAP and 0.1 mg/l IAA for shoot tip proliferation followed by sub culturing for up to 3 to 5 cycles for shoot multiplication. Shoots were individually transferred onto rooting medium (MS + IBA 5.0 mg/l).

In vitro salt stress

Well-rooted plantlets were selected and transferred onto MS medium supplemented with different levels of NaCl (50, 100, 150, 200 and 250 mM) while plants kept on MS medium without NaCl supplementation served as the control. All the treatments were repeated in triplicates and were maintained for 30 days after which observations were taken on growth, physiological and biochemical analyses.

Relative growth rate (RGR)

After one month, samples were weighed to calculate relative growth rate (RGR) as follows:

RGR= (final fresh weight – initial fresh weight) / initial fresh weight.

Membrane damage rate

The ionic concentration in the leaf sap was determined by measuring the electrolytic conductivity (mS cm^{-1}) of the sap with a portable EC/TDS meter (HANNA portable pH/conductivity/TDS meter, Sigma-Aldrich, USA) as described earlier (Bajji et al 2001).

Lipid peroxidation

Oxidative damage to the membrane lipids was estimated by analyzing the content of total thiobarbituric acid-reactive substances (TBARS), expressed as equivalents of malondialdehyde (MDA). The amount of MDA was calculated as described by Hichem et al. (2009).

Estimation of osmolytes and total soluble proteins

Free proline content was measured and determined according to Bates et al., (1973). About 500 mg of fresh leaves were homogenized in 3% (w/v) aqueous sulfosalicylic acid, and the residue was removed by centrifugation at 10,000 *g* for 10 min at 4°C. The supernatant (2 ml) was used for estimation. The amount of proline was determined from a standard curve using L-proline and expressed as µg of proline g^{-1} FW. Total soluble proteins (µg/FW) were assayed using the methodology of Bradford assay (Kruger, 1996) from the leaf samples (20 mg). Accumulation of GB in response to salt stress was determined according to Grieve and Grattan (1983) from fresh plantlets (500 mg). The GB content (µg g^{-1} FW) was determined from a standard curve prepared using glycine betaine (Sigma–Aldrich) as standard.

Na^+ and K^+ content

Determination of Na^+ and K^+ content was done by digesting 10 mg callus in concentrated HNO_3 in muffle furnace at 200°C prior to its re-digestion with H_2O_2 at 150°C. 1 ml deionised water was added for total solublization of the contents and 100 µl of this sample was then diluted to 5 ml with deionised water. The ionic content was measured on flame photometer and was expressed as µmol/g DW.

Antioxidant enzyme assays

The leaf samples (100 mg) were used for enzyme analysis. All the steps in preparation of the enzyme extract were carried out at 4°C. The samples were homogenized in 3 ml ice cold 50 mM sodium phosphate buffer (pH 7.0) including 0.1 mM EDTA and 1% (w/v) PVP in pre chilled mortar and pestle. The homogenate was centrifuged at 15,000 rpm for 20 min at 4°C. The supernatant was used as a crude enzyme extract for the antioxidant enzyme assays. The enzyme assays were performed at room temperature and the activities of the enzymes were determined with the spectrophotometer.

Guaiacol peroxidase (GPX) assay

1 ml assay system contained 50 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 10 mM guaiacol and 10 mM H_2O_2. Oxidation of guaiacol was monitored by following the increase in absorbance at 470 nm (E=26.6 $mM^{-1}cm^{-1}$) for 1 min at interval of 15 s after addition of 50 µl of crude enzyme The slope of readings between the time interval was considered as ΔA and the enzyme activity was expressed as mKat of CAT activity mg^{-1} protein.

Catalase (CAT) assay

CAT (EC 1.11.1.6) activity was measured by following the decomposition of hydrogen peroxide (H_2O_2) as described by

Cakmak and Marschner (1992) with minor modifications. The activity was measured in a reaction mixture (1 ml) containing 50 mM phosphate buffer (pH 7.0) and 15 mM H_2O_2. The reaction was initiated by adding 50 µl enzyme extract and the activity was determined as a result of H_2O_2 decomposition by monitoring the decrease in absorbance at 240 nm (E = 36, mM^{-1} cm^{-1}) for 2 min at an interval of 15 s. The slope of readings between the time interval considered as ΔA and the enzyme activity was expressed as mKat of CAT activity mg^{-1} protein.

Ascorbate peroxidase (APX) assay

APX (EC 1.11.1.11) activity was determined according to Nakano and Asada (1981). The reaction mixture (1 ml) contained 50 mM phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM H_2O_2, and 0.1 mM EDTA. The reaction was started by adding 50 µl of crude enzyme. Ascorbate oxidation was monitored for 1 min by measuring the decrease in absorbance at 290 nm at every 15 s (E = 2.8 mM^{-1} cm^{-1}). The enzyme activity was expressed as mKat of APX activity mg^{-1} protein.

Superoxide dismutase (SOD) assay

The total SOD (EC 1.15.1.1) was assayed according to Becana et al. (1986) by inhibition of the photochemical reduction of nitroblue tetrazolium (NBT). The reaction mixture (3 ml) contained 50 mM phosphate buffer (pH 7.8) and 0.1 mM EDTA to which an oxygen-generating system containing 14.3 mM methionine, 82.5 µM NBT, and 2.2 µM riboflavin, prepared freshly *in situ*, was added. The reaction was initiated by adding 50 µl of crude enzyme. The entire system was kept 30 cm below the light source (six 15-W fluorescent tubes) for 30 min. The reaction was stopped by switching off the light. For light blank, all the reactant without enzyme extract was incubated in light as for the samples, whereas all the reactants along with 50 µl enzyme extract were incubated in the dark for dark blank. The reduction in NBT was measured by monitoring the change in absorbance at 560 nm. The readings of light blank were used in calculation of enzyme units. 1 U SOD enzyme was defined as the amount that produces 50% inhibition of NBT reduction under the assay condition and expressed as U SOD activity mg^{-1} protein.

Statistical analysis

The experiment was laid out in a completely randomized design (CRD). The analyses were repeated with three independent biological samples. The data were analyzed by one-way analysis of variance (ANOVA) using the statistical software SPSS 10.0, and the treatment means were compared by using Duncan's multiple range test (DMRT) at $P \leq 0.05$. Data were expressed as mean ± standard error (SE).

RESULTS AND DISCUSSION

It was observed that as the concentration of NaCl increased, the RGR was found to decrease by 42.1 and 77.7% in CoC 671 and Co 86032, respectively. Incidentally, most significant decline in RGR was found at 100 mM for CoC 671 compared to Co 86032, at 50 mM (Figure 1a).

It was seen that as the NaCl concentration increased, the relative electrolytic leakage in the plantlets also increased gradually which is directly related to MDR. The MDR was highest at 100 mM for both the varieties; however, Co 86032 was found to be more affected than CoC 671. It was also observed that the membrane damage rate was unexpectedly lower at 150 mM. Further down the gradient, the MDR seemed almost constant due to the possibility of a high lethality in the plant cells leading to inadequacy in release of ions in the solution (Figure 1b). It has been reported that the gradual increase in salt stress elevates the MDR. This is due to the formation of reactive oxygen species (ROS), especially, superoxide (O_2^-) and singlet oxygen ($^1O_2$) in the cell which oxidize the membrane lipids and structural proteins of cell membrane (Lin and Kao, 1999; Hasegawa et al; 2000; Bor et al., 2002; Patade and Suprasanna, 2009). This result in the augmentation of peroxidation of membrane lipids (Figure 1c) which display a gradual increase when their peroxidation was quantified by the amount of malondialdehyde (MDA) accumulated due to peroxidation of cell membrane. It was observed that MDA level increased gradually in both varieties up to 150 mM of NaCl stress wherein an increase of 32.5 and 55.8% was observed in CoC 671 and Co 86032, respectively.

Both varieties displayed a significant rise in the level of proline. About 43% increase was seen in CoC 671 as compared to 189% in Co 86032 (Figure 1d). Among the varieties, the differential accumulation of proline may be due to the response of a variety towards the environment (Hosseini et al., 2010). Although it has been shown by various groups that over accumulation of proline is non-specific in nature towards stress (Hosseini et al., 2010; Errabii et al., 2007 and Ashraf and Harris, 2004), it could also be due to higher amount of cell injury, thus proving them to be salt susceptible (Errabii et al., 2007; Lu et al., 2007; Aazami et al., 2010). The overproduction of proline may also mean a greater stress impact in Co 86032 as compared to CoC 671, thus, rendering higher salt tolerance in CoC 671 than Co 86032. Proline has also been reported to accumulate in order to maintain osmotic potential of the plant cell under stress. Proline also acts as a neutral storage chemical for carbon and nitrogen in the cell without affecting other molecules or enzymes enabling tolerance of cells towards salts (Stewart and Lee 1973; Greenway and Munns, 1980). Additionally, it also acts as scavenger of free radicals, thus, buffering the redox cell conditions, besides acting as protein hydrotope thereby lowering cytoplasmic acidosis, and maintaining required $NADP^+$/NADPH ratios compatible with metabolism (Ashraf and Foolad, 2007).

Similar to the proline, the levels of proteins and glycine betaine were also found to accumulate substantially as salt stress was increased up to 150 mM, beyond which, the levels gradually dropped in Co 86032, while it sparsely reduced in CoC 671 (Figure 1e and f). Glycine betaine, like other metabolites, was also found to increase more than twice, from non tolerant conditions to tolerant conditions up to 150 mM of NaCl , beyond

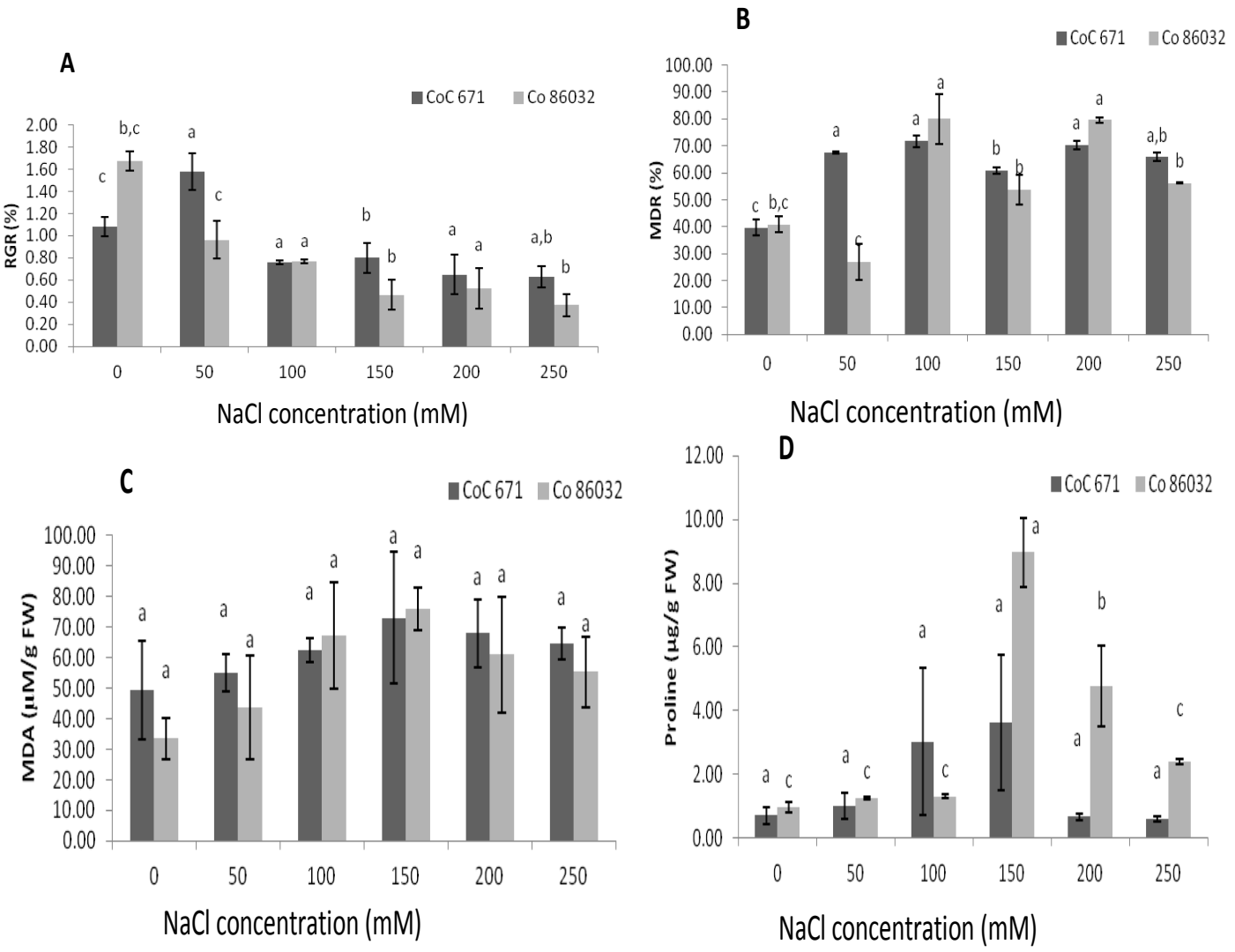
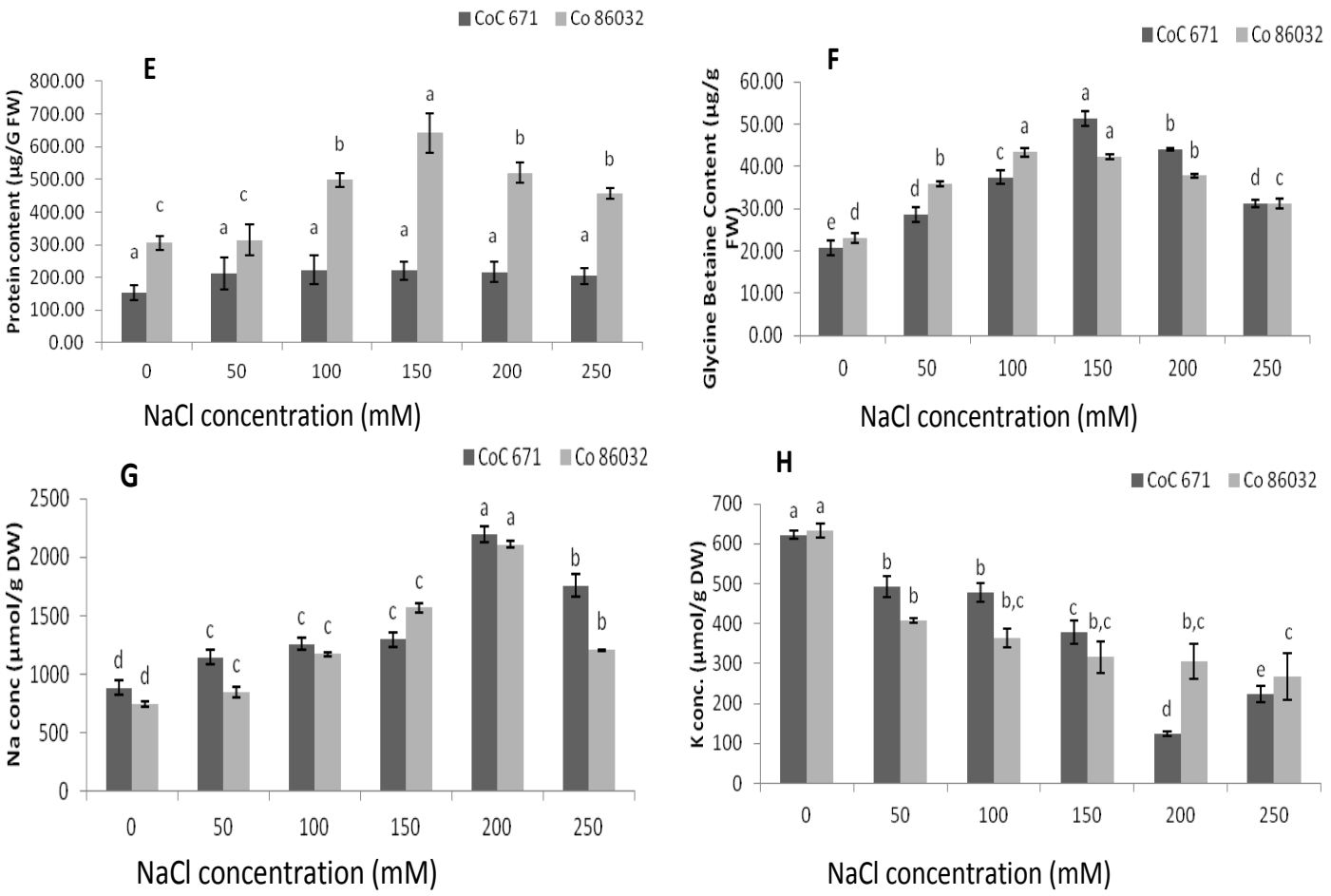


Figure 1. Effects of NaCl stress on the physiological and biochemical responses in sugarcane; a) relative growth rate, b) membrane damage rate, c) MDA activity, d) free proline, e) protein, f) glycine betaine, g) Na^+ content, h) K^+ Content, i) guaiacol peroxidase, j) Catalase, k) Ascorbate peroxidase, l) superoxide dismutase, in CoC 671 and Co 86032. Each value represents mean of three replications with each vertical bar being standard error ($P< 0.05$).

which,there was a decline, possibly due to considerable lethality in both varieties. In contrast, GB accumulation in CoC 671 was found to be about 5% higher in CoC 671 than Co 86032; with Co 86032 only at the threshold concen-tration of salt (Figure 1f).

Glycine betaine is a quaternary ammonium compound having the primary chain supplemented with methylated nitrogen (McNeil et al., 1999) and has been reported to accumulate in the plantlets undergoing salt stress. It has been found not only acting as a compatible osmolyte, but also as an osmoprotectant. The molecule is chloroplastic in origin and is responsible for maintaining photosynthetic efficiency during salt stress, besides having a protective ability against heat or cold shock (Ashraf and Harris, 2004; McNeil et al., 1999). The molecule has been reported to decrease the water potential in the cells during the stress conditions resulting in delayed wilting in tolerant varieties as against their susceptible counter-parts.

The accumulation of sodium and potassium was observed to be higher in CoC 671 as compared to Co 86032. Sodium accumulation increased to a significant level as the NaCl stress increased and reached a peak at 200 mM NaCl, which was in contrast to other parameters which gave their peak activity or level at 150 mM NaCl stress. It might have been due to the accumulation and arrest of NaCl in the dead tissues of the plantlets. Similarly, a significant drop was seen in the potassium level as the salt stress increased whereas the sodium concentration increased by about 2.5 and 2.84 times, respectively, while potassium concentration depleted by 2.77 and 2.36 times, respectively in both varieties (Figure



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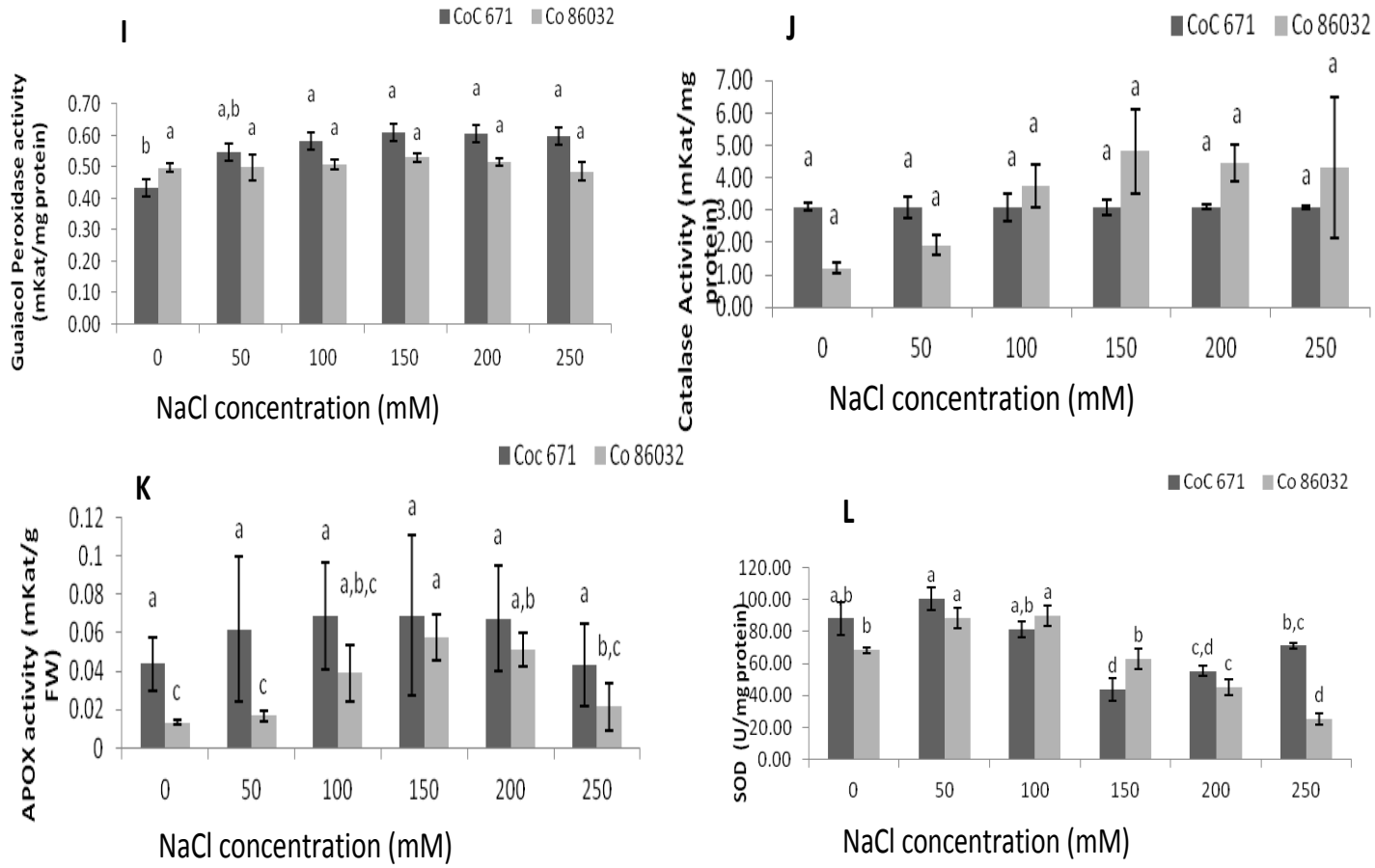
1g and h). During the NaCl stress, the accumulation of Na^+ increased considerably, thus unbalancing the ionic equilibrium, causing K^+ ions to escape out of the cell. The influx of Na^+ is particularly dangerous to cell as numerous processes like protein synthesis, osmotic potential maintenance and enzymatic activities are K^+ concentration based and are disrupted in its absence (Li et al., 2010). It has also been reported that the tolerant varieties efficiently compartmentalize Na^+ than the susceptible ones (Errabii et al., 2006), based on which it may be said that CoC 671 was adapted to a higher Na^+ concentration (150 mM) than Co 86032. As for the K^+ accumulation, it displayed similar patterns as Na^+ accumulation and displayed a higher concentration in CoC 671 than Co 86032 in stressed conditions, as was reported in many previous works done (Errabii et al., 2006; Li et al., 2010)

Salt stress induces cellular accumulation of ROS which can damage membrane lipids, proteins and nucleic acids (Mansour et al., 2005; Ben Amor et al., 2007). A correlation between the antioxidant capacity and NaCl tolerance has been demonstrated in some plant species and it has been pointed out that salt-tolerant species increased their antioxidant enzyme activities and antioxidant contents in response to salt treatment, whereas salt-sensitive species failed to do so (Shalata et al., 2001; Demiral and Türkan, 2005).

The enzymatic activity of guaiacol peroxidase showed a gradual increase in both varieties till 150 mM NaCl stress after which it displayed almost a plateau with minor decrease (Figure 1i). In the control samples, the enzyme activity was higher in Co 86032; however, when the NaCl stress was applied, the activity was found to be higher in CoC 671. Similarly, the activity of catalase was observed to increase gradually with respect to increase in NaCl concentration. The activity significantly rose from about 5 times in 150 mM NaCl supplemented medium as compared to the control sample (Figure 1j). The activity, however, depleted after this concentration as the lethality of plants increased. However, the activity of catalase was in total paradox with other stress-related parameters like guaiacol peroxidase and membrane damage rate which were comparatively higher in CoC 671 than Co 86032. However, contrastingly, the catalase activity rate was significantly higher in Co 86032. Interestingly, the



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catalase activity in CoC 671 appeared to be almost constant across the NaCl concentrations, indicating that the variety is tolerant towards NaCl stress. Ascorbate peroxidase also displayed a similar trend; the only difference was that a higher activity was observed in CoC 671 compared to Co 86032 at 150 mM NaCl, beyond which the activity showed a decrease (Figure 1k).

The activity of superoxide dismutase (SOD) also followed a similar pattern; the only difference was an increase in activity found at 100 mM instead of 150 mM of NaCl stress. The SOD activity in CoC 671 was significantly higher than in Co 86032 at 150 mM (Figure 1l).

Guaiacol peroxidase belongs to a large family of peroxidases which are activated in the presence of H_2O_2 so as to alleviate the superoxide (O_2^-) and singlet oxygen ($^1O_2$). The enzyme is classified in the group of antioxidants, whose other members include superoxide dismutase (SOD), glutathione reductase, and catalase (Sairam et al., 2002). It has been reported that the reactive oxygen species (ROS) increase with the increment in salt stress (Xiong et al., 2002; Ashraf and Harris, 2004). Thus, as the accumulation of antioxidants increased significantly, so did the plant's tolerance to salt stress (Ashraf, 2004; Ashraf and Harris, 2004; Piza et al., 2003; Sairam et al., 2002). Therefore, there is a significant activity of enzymes like catalase (Gosset et al., 1996) and guaiacol peroxidase (Mika and Lütje, 2003) in salt tolerant varieties as compared to salt susceptible ones. In several plants, the activity of catalase showed a significant decrease under stress conditions, possibly due to generation of salicylic acid in the cytoplasm, which acts as an inhibitor of the enzyme (Shim et al., 2002). Such a possibility cannot be precluded for the low accumulation of catalase in CoC 671 as against Co 86032, in spite of displaying a higher salt tolerance.

The enzyme activity of the APX in the plants subjected to saline conditions suggests that oxidative stress is an important component of salt stress (Stepien and Klobus, 2005). In this study, several folds of higher activity of APX were seen in Coc 671 compared to Co 86032. Stepien and Klobus (2005) proposed that the APX of the more active ascorbate-glutathione cycle may be related to the development of relatively higher salt tolerance in maize. Superoxide dismutase is considered as the first line of defense against ROS as it converts a very highly reactive superoxide ion (O_2^-) (Jebara et al., 2005; Bor et al., 2002). The tomato under high salt concentration showed

higher antioxidant enzyme activities such as SOD, catalase, ascorbate peroxidase, glutathione reductase, and GST (Rodriguez-Rosales et al., 1999). The activity of the enzyme increases significantly with the increment in NaCl stress (Bor et al., 2002), thus regulating the level of other peroxidases necessary for preventing peroxidation of organelle and cell membrane (Lin and Kao, 1999).

Accumulation of organic solutes/osmolytes augments the osmotic adjustment, which is critical for physiological machinery for achieving tolerance to biotic and abiotic stresses. Pro synthesis and antioxidant system may be responsible for higher tolerance in CoC 671. In sugarcane, growth reduction and reduced viability were associated with an increase in Na^+ but a corresponding decline in K^+, suggesting that sugarcane has a typical glycophytic nature. Our results suggest that the salt ion accumulation and osmolytes buildup may have an important role in osmotic adjustment in sugarcane under salt stress.

Conclusions

It was observed from the physiological parameters (not discussed here) and the biochemical parameters that CoC 671 shows higher tolerance towards NaCl than Co 86032 and it was found to survive 150 mM of NaCl induced salinity stress compared to Co 86032, which showed tolerance only up to 100 mM NaCl. Further, the study also suggests that *in vitro* cultured tissues or cells and plantlets can be useful as a system to screen for salinity stress in sugarcane.

ACKNOWLEDGEMENTS

The work was carried out in the DAE-BRNS project from BARC, Mumbai, India. We are also grateful to the Director General and Director, Agricultural Science and Technology, VSI for providing all facilities. Ashok A. Nikam is thankful to Dr. V Ghole, VSI and Dr S. S. Sheelavantmath, Department of Biotechnology, Sinhgad College of Science, Pune, for suggestions in improving the manuscript.

Abbreviations

BAP, Benzyl amino purine; **Kin,** kinetin; **MS,** Murashige and Skoog basal medium; **IAA,** indole-3-acetic acid; **IBA,** indole-3-butyric acid; **RGR,** relative growth rate; **MDR,** membrane damage rate; **MDA,** malondialdehyde; **GB,** glycine betaine; **CAT,** catalase; **APX,** ascorbate peroxidase; **GPX,** guaiacol peroxidase; **NaCl,** sodium chloride; **ROS,** reactive oxygen species; **EDTA,** ethylene diamine tetra acetic acid; **PVP,** poly vinyl pyrrolidine; **ANOVA,** analysis of variance.

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