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RESEARCH ARTICLE

Exogenous Application of Proline Alleviates Salt Induced Oxidative Stress More Efficiently than Glycine Betaine in Sugarcane Cultured Cells

Vikas Yadav Patade · Vinayak Haribhau Lokhande · Penna Suprasanna

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Abstract Antioxidant defense system provides protection against oxidative damage caused by abiotic stresses including salinity. Ameliorative effects of L-proline, L-glutamine, glycine betaine (GB) on growth, proline accumulation and antioxidant enzyme activities were studied using cultured cells of sugarcane against salt (NaCl) stress. NaCl stress reduced growth rate significantly over the control however, proline or glutamine supplementation resulted in growth revival. Proline supplementation to media with or without salt increased accumulation of free proline significantly than the controls and other (proline, GB and glutamine) treatments. Salt stress led to increase in superoxide dismutase (SOD) and glutathione reductase activity whereas guaiacol peroxidase (GPX), catalase and ascorbate peroxidase activities were significantly suppressed. Proline supplementation to the salt medium improved the GPX activity over the salt media supplemented with glutamine or glycine betaine. The activity ratio between SOD and H_2O_2 scavenging enzyme activities, which is considered as a working hypothesis for biochemical marker for salt tolerance, was lower in salt medium supplemented with proline. Thus, the higher growth rate and the lower activity ratio suggest maximum salt stress ameliorative potential of proline in sugarcane cultured cells.

V. Y. Patade · V. H. Lokhande · P. Suprasanna (✉)
Functional Plant Biology Section, Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Trombay, Mumbai 400085, India
e-mail: penna888@yahoo.com

Present Address:
V. Y. Patade
Molecular Biology and Genetic Engineering Division, Defence Institute of Bio-Energy Research, Goraparao, Haldwani, Nainital 263139, India

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Abbreviations

RGR	Relative growth rate
MS	Murashige and Skoog basal medium
2,4-D	2,4-Dichlorophenoxy acetic acid
GB	Glycine betaine

Introduction

Sugarcane (*Saccharum* species hybrid) is one of the important agro-industrial sugar crops of the world, cultivated in more than 20 million hectares, a little about 2 % of the total cropped area. With increased salinization of soil and water, abiotic stress is assuming as a major limiting factor for plant growth and will soon become even more severe as desertification covers more and more of the world's terrestrial area. Sugarcane being a glycophyte, high levels of salt in the soil affects growth rate, cane yield as well as the sucrose content in cane (Wahid 2004).

Salt stress affects plants by causing ion imbalance and hyper-osmotic stress, which often led to oxidative damage. It is now well documented that, reactive oxygen species (ROS) production is increased under saline conditions and their higher concentrations in the absence of any protective mechanism seriously disrupt normal metabolism of plants through oxidation of membrane lipids, proteins and nucleic acids (Noctor and Foyer 1998; Mantri et al. 2011). Plant species are endowed with a defence system against the harmful effects of the ROS, which involves antioxidants, reductants and antioxidant enzymes such as catalase

(CAT), glutathione reductase (GR), ascorbate peroxidase (APX) and superoxide dismutase (SOD). Salinity stress cause qualitative as well as quantitative alterations to the antioxidant systems, which are often related to the levels of resistance to the salinity (Parida and Das 2005). Further, over or reduced expression of antioxidant enzyme encoding genes through plant genetic engineering has provided the supporting evidence on the involvement of antioxidant enzymes in salt tolerance (Torsethaugen et al. 1997).

Plants when confronted with salinity stress, respond with a significant accumulation of compatible solutes in the cytosol without disturbing intracellular biochemistry, which ameliorate the detrimental effects of salinity. The elevated levels of the osmolytes accumulated in plant cells correlate with enhanced stress tolerance through scavenging free radicals and protecting enzymes (Dionisio-Sese and Tobita 1998; Sharma and Dubey 2005). In earlier studies, it has been demonstrated that exogenous application of osmolytes like proline and glycine betaine (GB) mitigate the detrimental effects of salinity (Harinasut et al. 1996). However, the mechanisms underlying such amelioration are not fully understood. Hoque et al. (2007) studied the effect of exogenous application of these osmolytes on the activities of ascorbate–glutathione cycle enzymes (APX, DHAR and GR).

The in vitro culture technique provides a controlled and uniform environment for studying physiological and biological processes in plants of particularly salt induced ionic and osmotic stresses at cellular level (Ahmad et al. 2007). Studies with other glycophytes have shown that the degree of salt tolerance observed in whole plant is also exhibited in callus tissue suggesting that salt tolerance mechanism operates at cellular level (Arzani 2008). For an effective and commercial exogenous application of these osmolytes, the mechanism of actions and the most optimal concentrations must be carefully determined. The present investigation was attempted to study the ameliorative role of L-proline, GB and L-glutamine in salt (NaCl) treated sugarcane cells.

Materials and Methods

Embryogenic callus cultures of sugarcane cv. Co 86032 were established from spindle leaf explants (Patade et al. 2008) on callus induction medium (control-1 medium; MS basal salts supplemented with 100 mg l^{-1} malt extract, 100 mg l^{-1} L-glutamine, 1,000 mg l^{-1} casein hydrolysate, 1 mg l^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D) and 30 g l^{-1} sucrose, gelled with 0.2 % gel rite). The cultures were maintained through regular subculture on control-1 medium under a 16 h photoperiod (30 µmol m^{-2} s^{-1} PFD) at 25 ± 2 °C and 70 % RH. Embryogenic calli (500 mg) were cultured on control-1, control-2 (control-1 without malt extract, L-glutamine and casein hydrolysate), control-2 + salt, and control-2 medium supplemented with 20 mM of L-proline, L-glutamine and GB with or without 100 mM salt. The control-2 was considered in order to study the stress ameliorative effect of individual bioregulatory molecules. The growth (mg) was determined on fresh weight (FW) basis in terms of relative growth rate (RGR) after 1 month's culture on these treatments with or without NaCl. The RGR was calculated using formula: RGR = (final fresh weight − initial fresh weight)/initial fresh weight. The dry weight (DW) was measured by drying 1 g of calli at 55 °C for 72 h. The water content (WC) was calculated as WC (%) = (FW − DW)/FW × 100.

Free proline content was determined according to Bates et al. (1973). Callus tissue (500 mg) was homogenized in 3 % aqueous sulfosalicylic acid. The filtered homogenate (2.0 ml) was reacted with 2.0 ml each of acid ninhydrin and acetic acid at 100 °C for 1 h, and the reaction was terminated in an ice bath. The reaction mixture was extracted with 4.0 ml toluene and mixed vigorously with a stirrer for 10–15 s. The absorbance of the chromophore containing toluene was recorded at 520 nm using toluene as a blank. The free proline concentration (µg g^{-1} FW) was determined using a standard curve.

Callus samples (200 mg) stressed for 30 days were homogenized in 3.0 ml ice cold 50 mM sodium phosphate buffer (pH 7.0) including 0.1 mM EDTA and 0.1 % (w/v) PVP in pre chilled mortar and pestle. An appropriate aliquot/dilution of the supernatant was used as a crude enzyme(s) for the antioxidant enzyme assays. Protein content of the extract was determined by the Bradford (Bradford 1976) method using BSA-bovine serum albumin as a standard. All the enzyme activity assays were performed at room temperature with a UV–Visible spectrophotometer (UV-1700 PharmaSpec, UV–Visible spectrophotometer, Shimadzu, Japan).

The specific superoxide dismutase (SOD, EC 1.15.1.1) SOD activity was assayed in terms of inhibition of the photochemical reduction of NBT-nitroblue tetrazolium (Beyer and Fridovich 1987). Glutathione reductase (GR, EC 1.6.4.2) was determined by monitoring the glutathione-dependent oxidation of NADPH at 340 nm ($\varepsilon = 6.2$ mM^{-1} cm^{-1}), according to the modified method of Foyer and Halliwell (1976). Guaiacol peroxidase (GPX, EC 1.11.1.7) specific activity was measured in the assay system consists of 50 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 10 mM guaiacol and 10 mM H_2O_2 in cuvette. Ascorbate peroxidase (APX, EC 1.11.1.11) activity (mKat mg^{-1} protein) was determined according to Nakano and Asada (1981). Catalase assay (CAT, EC 1.11.1.6) activity was measured by following the decomposition of hydrogen peroxide as described by Cakmak and Marschner (1992) with some modifications.

The treatments and controls of the experiments were replicated four times. CropStat for Windows (7.2.2007.2 module), developed by the Biometrics unit, IRRI, Philippines was used for analysis of variance (ANOVA) for experiments laid out in completely randomized design (CRD). The treatment means were compared by using the Duncan's Multiple Range Test (DMRT) at a significance level of $P \leq 0.05$.

Results

Salt treatment reduced growth rate significantly as compared to the control-1 (Table 1). However, the differences in the growth rate of cells growing on salt and control-2 media were at par. Proline supplementation in salt medium improved growth rate significantly (by 1.5-fold) over the salt treatment without proline. However, the growth rate in proline treatment was significantly lower than both the controls as well as the treatment with salt + proline (7.9-fold lower). The growth rate in glutamine or GB supplemented medium with or without salt was also significantly higher than the control. The increased growth rate in treatment with L-glutamine was associated with significantly increased WC over the control. DW was significantly higher (188 mg g^{-1} FW) in treatment with proline than with both the controls and lower (142 mg g^{-1} FW) in control + salt (Table 1). In rest of the treatments, DW was at par with the control. Protein content was significantly higher in the control (3.08 mg g^{-1} FW) than rest of the treatments. Proline content was significantly higher in proline supplementation with (710.7 μg g^{-1} FW) or without salt (814.9 μg g^{-1} FW) media. Though, proline accumulation was more in salt treatment (43.4 μg g^{-1} FW) than the control-1 (29.11 μg g^{-1} FW), surprisingly the accumulation was lower than the control without salt (66.2 μg g^{-1} FW). In general, proline accumulation was more in treatment with amino acids supplementation and salt, than the treatment without salt (Table 1).

SOD specific activity was significantly higher in control-2 + salt treatment (369.22 U mg^{-1} protein) than the control-1 (114.33 U mg^{-1} protein), control-2 (169.94 U mg^{-1} protein) and rest of the treatments. In proline, GB and glutamine supplemented salt medium, SOD activities were higher than the respective proline, GB and glutamine treatment without salt except with GB (Fig. 1). The treatment with proline without salt significantly reduced the SOD activity (41.43 U mg^{-1} protein) which was 24 % of control-2. GR specific activity (mKat mg^{-1} protein) was the highest in control-2 + salt treatment (6.6-fold of the control-1) followed by the GB (6.5-fold over the control-1) treatment (Fig. 2). In general, the GR activity was higher in the salt treatments with proline, GB and glutamine than the respective treatments of proline, GB and glutamine without salt except GB. GPX activity was higher in control-1 (10.1 mKat mg^{-1} protein) and control-2 (25.78 mKat mg^{-1} protein) compared to the other treatments (Fig. 3). The GPX activity reduced drastically in the rest of the treatments (by 37–91 % over the control-1 and by 76–97 % over the control-2). In general, the activity of GPX was higher in treatment without salt (only proline, GB and glutamine) than the respective treatment with proline, GB and glutamine + salt. However, the trend was reverse in proline (Fig. 3). Salt medium supplemented with proline increased the GPX activity to 63 % of the control-1, whereas the activity was only 40 % of the control-1 in control-2 + salt medium. APX activity was also significantly higher in control-1 (60.37 mKat mg^{-1} protein) and control-2 (47.35 mKat mg^{-1} protein) than the rest of the treatments. In general, the APX activity was higher in a particular treatment without salt than the respective proline, GB and glutamine treatment + salt except, proline, and glutamine (Fig. 4) supplemented media. Salt stress led to significant inhibition of CAT activity similar to GPX and APX. CAT activity was significantly higher in control-1 (3.93 mKat mg^{-1} protein) and control-2 (5.22 mKat mg^{-1} protein) than the rest of the treatments. Among these, CAT activity in the proline, GB and glutamine + salt treatments was lower than the respective treatment without salt. However, in case of supplementation with L-glutamine, the activity was higher in presence of salt (0.24 mKat mg^{-1} protein) than the respective treatment without salt (Fig. 5). The activity ratio between SOD and H_2O_2 scavenging enzymes was the highest (11.46) in the salt treatment. However, supplementation of proline, GB and glutamine with or without salt reduced the ratio. Among the salt treatments supplemented with proline, GB and glutamine, the ratio was the lowest in salt medium supplemented with proline. Whereas the activity ratio among the salt + proline, Gb and glutamine treatments was higher in L-glutamine (9.2) or GB (6.73) supplemented salt media. SOD: GPX ratio, among the proline, GB and glutamine treatments with salt was lowest in salt medium supplemented with proline.

Discussion

In the present study, salt stress reduced the growth of cultured sugarcane cells significantly over the control (growing on control-1 medium). This growth limitation is possibly due to salinity stress may presumably be due to the osmotic effect of salt in earlier phase or the ionic stress which, impacts growth much later (Munns and Tester 2008). The exogenous application of proline under salt treatment increased the internal levels of this imino acid and enhanced the growth rate of cells by 1.5-fold than the

Table 1 Effect of salt stress and other proline, glycine betaine and glutamine treatments on growth, protein and free proline accumulation in sugarcane cultured cells

Treatments	RGR	Water content (%)	Dry Wt. (mg g^{-1} FW)	Protein content (mg g^{-1} FW)	Proline (μg g^{-1} FW)
Control-1	6.91^{a}	$83.5^{b,c}$	$166^{b,c}$	3.08^{a}	29.11^{d}
Control-2	3.54^{e}	$84.7^{b,c}$	154^{d}	1.19^{e}	66.2^{c}
Control-2 + NaCl	$3.72^{d,e}$	85.8^{b}	142^{e}	0.66^{f}	43.4^{d}
Control-2 + proline	0.72^{f}	81.3^{c}	188^{a}	2.60^{b}	814.9^{a}
Control-2 + proline + NaCl	5.65^{b}	$84.5^{b,c}$	$156^{c,d}$	1.62^{d}	710.7^{b}
Control-2 + L-glutamine	5.75^{b}	91.5^{a}	86^{f}	2.09^{c}	34.5^{d}
Control-2 + L-glutamine + NaCl	5.08^{c}	86.7^{b}	134^{e}	$1.84^{c,d}$	70.3^{c}
Control-2 + GB	5.14^{c}	$83.3^{b,c}$	168^{b}	$0.85^{e,f}$	6.5^{e}
Control-2 + GB + NaCl	3.85^{d}	$84.7^{b,c}$	154^{d}	2.60^{b}	41.5^{d}
SE (M)	0.13	1.32	4	0.13	4.6
LSD	0.37	4	11	0.41	13.3

The values indicate means of four observations. Means superscribed by different alphabets in each column were significantly different at $P \leq 0.05$, according to DMRT

Control-1 medium MS basal salts supplemented with 100 mg l-1 malt extract, 100 mg l-1 L-glutamine, 1,000 mg l-1 casein hydrolysate, 1 mg l-1 2,4-D and 30 g l^{-1} sucrose, gelled with 0.2 % gel rite; *Control-2 medium* control-1 without malt extract, L-glutamine and casein hydrolysate

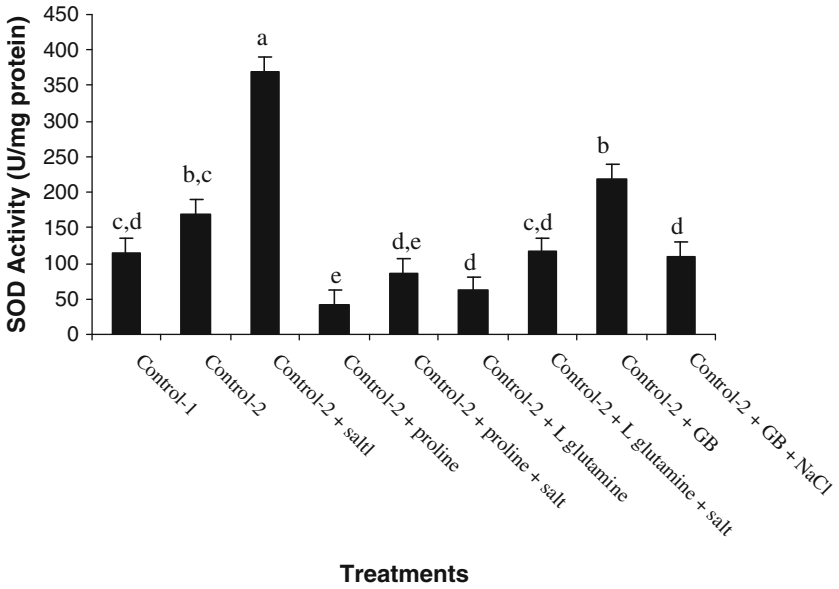

Fig. 1 SOD activity as affected by salt stress and other proline, glycine betaine and glutamine treatments in sugarcane calli. *Column bars* indicate means of four observations. Means super scribed by *different alphabets in each column* were significantly different at $P \leq 0.05$, according to DMRT. *Error bars* indicate SE (M)

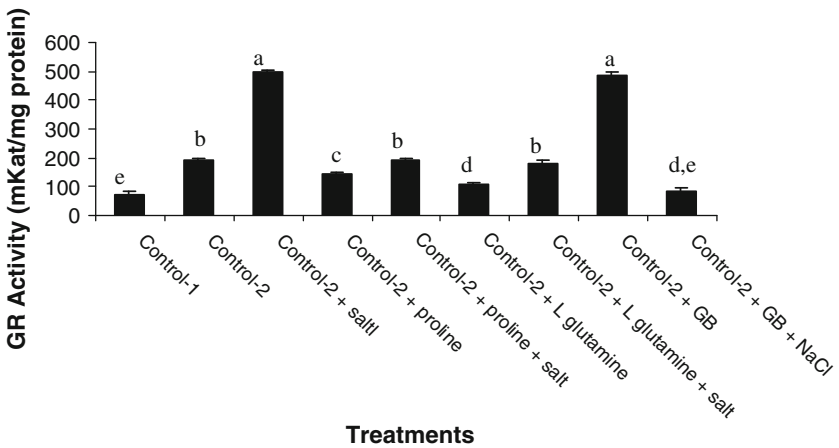

Fig. 2 GR activity as affected by salt stress and other proline, glycine betaine and glutamine treatments in sugarcane calli. *Column bars* indicate means of four observations. Means super scribed by *different alphabets in each column* were significantly different at $P \leq 0.05$, according to DMRT. *Error bars* indicate SE (M)

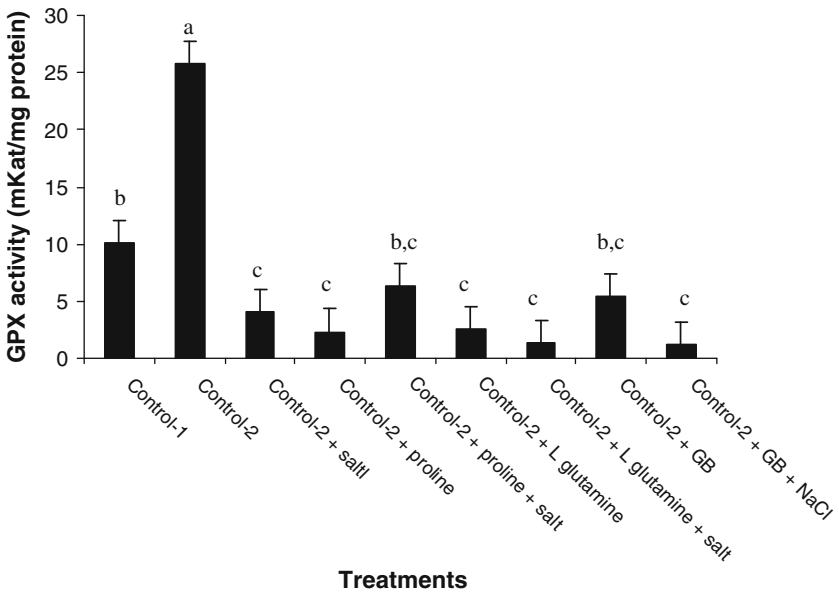

Fig. 3 GPX activity as affected by salt stress and other proline, glycine betaine and glutamine treatments in sugarcane calli. *Column bars* indicate means of four observations. Means super scribed by *different alphabets in each column* were significantly different at $P \leq 0.05$, according to DMRT. *Error bars* indicate SE (M)

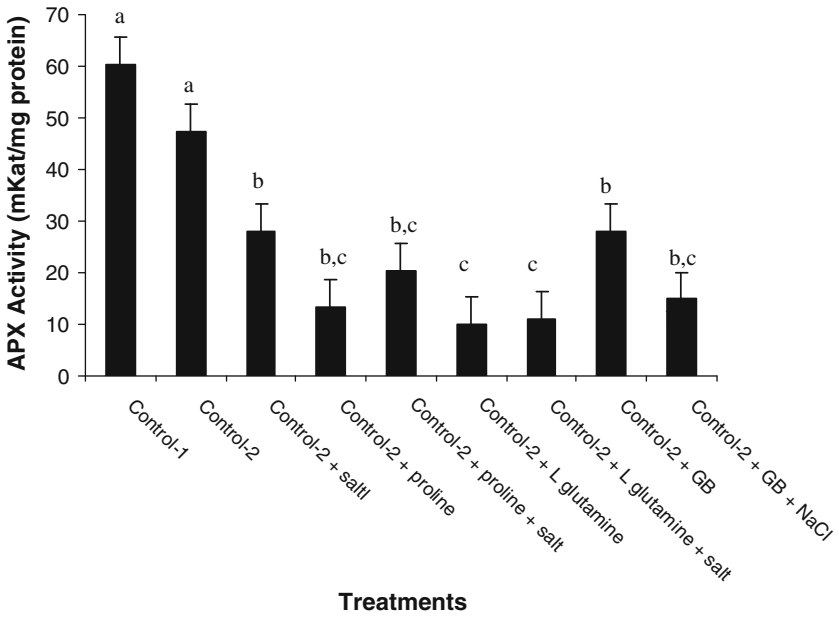

Fig. 4 APX activity as affected by salt stress and other proline, glycine betaine and glutamine treatments in sugarcane calli. *Column bars* indicate means of four observations. Means super scribed by *different alphabets in each column* were significantly different at $P \leq 0.05$, according to DMRT. *Error bars* indicate SE (M)

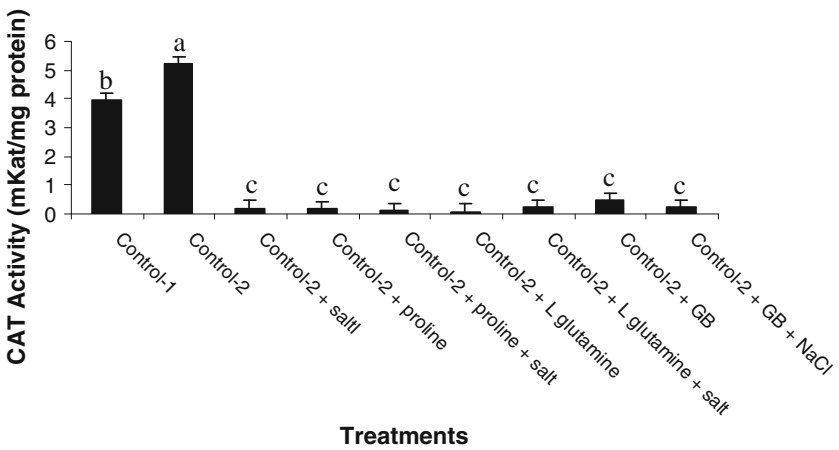

Fig. 5 CAT activity as affected by salt stress and proline, glycine betaine and glutamine treatments in sugarcane calli. *Column bars* indicate means of four observations. Means super scribed by *different alphabets in each column* were significantly different at $P \leq 0.05$, according to DMRT. *Error bars* indicate SE (M)

salt minus proline treatment. However, the growth rate of the cells cultured on proline-supplemented medium without salt was significantly lower than the control. This may be due to excessive accumulation or application of proline. The excessive application of proline in plants is known to inhibit growth and affects many cellular metabolisms adversely (Nanjo et al. 2003). The excessive exogenous application of proline is hypothesized to cause damage to ultra-structures of chloroplast and mitochondria (Hare et al. 2002). Such loss of organellar integrity can result in a significant increase in reactive oxygen intermediates in chloroplast and mitochondria, causing considerable reduction in growth rate. Therefore, it is essential to determine optimal concentrations of proline, which vary depending on species and genotype. Supplementation of GB to medium with or without salt resulted in higher growth rate as compared to the salt treatment alone (control-2 + salt). Accumulation of compatible solutes like proline and GB under salt stress is hypothesized to increase salt tolerance of plants (Gorham 1995; Harinasut et al. 1996; Nakamura et al. 1997; Patade et al. 2008). The improved growth rate due to GB supplementation under salt treatment may be due to reduced salt induced ultra-structural damages (Yang and Lu 2005). However, the salt medium supplemented with proline exhibited more growth rate than that of GB. Hoque et al. (2007) also concluded that though both proline and GB mitigate the detrimental effects of salt stress, proline is more effective than the later. Supplementation of GB in salt medium increased the protein content as compared to the salt stress alone. Similar results were reported by Demiral and Turkan (2006) based on study on exogenous application of GB in salt tolerant and salt-sensitive rice cultivars. The authors concluded that the exogenous GB application reduced the impact of salt-stress on growth and senescence, through enhanced photosynthetic efficiency and increased the protein content.

Antioxidant enzymes especially CATs play an important role in scavenging of ROS in the cell through detoxification of H_2O_2. Since, CAT activity was not enhanced during the treatments with salt and various bioregulatory compounds, other classical metabolic substances might have been involved in free radical scavenging such as ascorbate, reduced glutathione (Noctor and Foyer 1998; Imlay 2003). Plants with low activities of both CAT and cytosolic APX show less severe stress symptoms than plants that lack either one of these enzymes (Rizhsky et al. 2002). In the present study, GR activity was significantly higher in salt treatment over the controls. However, the enhanced activity in GR was not associated with increase in growth rate of cells. Thus, it appears that GR activity alone may not be sufficient to efficiently scavenge the ROS and reduce the oxidative damages in sugarcane cells. APX activity was significantly higher in controls than the rest of the treatments; on the contrary, SOD activity was significantly higher in salt treatment. Chagas et al. (2008) also reported imbalance between the specific activities of SOD and APX enzymes with response to oxidative stress in sugarcane. Experimental evidence has proven the susceptibility of APX under the oxidative stress conditions than the other antioxidant enzymes (Chagas et al. 2008). In addition, APX is the first enzyme that inactivate after short exposure of light (Miyake et al. 2006). Results of the present study also showed the higher susceptibility of APX in salt stress. The APX activity was significantly lower in all the other treatments than the control. Similar results were also reported in isolated tobacco chloroplasts, where APX activity did not exhibit any significant increase under oxidative stress (Miyake et al. 2006). Our data indicated that APX activity was higher in treatment without salt than the respective treatment with salt except in proline supplemented media. Demiral and Turkan (2004) also concluded that exogenous GB application protect tolerant as well as sensitive rice seedlings from salinity-induced oxidative stress through altered antioxidant enzyme activities, which lowered the lipid peroxidation levels of both the cultivars under salt treatment.

The higher efficiency of the antioxidant-enzymatic system of plants has been reported in tolerant sorghum genotype (CSF 20) and hypothesized to be responsible for salt tolerance mechanism (da Costa et al. 2005). The authors proposed the activity ratio of SOD and H_2O_2 scavenging enzyme as a working hypothesis for biochemical marker for salt tolerance. The higher activity ratio was reported to be associated with the sensitive response. In the present study, the ratio was low in controls and high in the salt treatment. However, supplementation with proline had the lowest activity ratio than with L-glutamine or GB. Beside this, the callus growth rate was significantly high in proline-supplemented salt medium, indicating the better ameliorative potential of proline than the later in sugarcane-cultured cells in vitro. The results are in agreement with an earlier report (Hoque et al. 2007; Kaya et al. 2007). The stress ameliorative potential was not only due to being an osmolyte but also its free radicals scavenging ability through enhanced antioxidant activity as revealed in the present study, and maintaining the cellular redox potential under stress conditions. Besides, the rapid breakdown of proline upon relief of stress often provides sufficient reducing agents to support mitochondrial oxidative phosphorylation to generate ATP for alleviating stress induced damages (Hare and Cress 1997). Proline is also known to induce expression of salt stress responsive genes that possess proline responsive elements in their promoters (Chinnusamy et al. 2005). In conclusion, the results strongly supports that the exogenous application of biological molecules like proline imparts tolerance to salt

stress at cellular level in sugarcane. Further, for an effective, commercial exogenous application of these compounds, the mechanism of action and the most optimal concentrations must be carefully determined at plant level under field conditions.

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