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Effects of cadmium on antioxidant enzyme activities in sugar cane

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Abstract

Sugar cane (*Saccharum officinarum* L. cv. Copersucar SP80-3280) seedlings were grown in nutrient solution with varying concentrations (0, 2 and 5 mM) of cadmium chloride for 96 h. Leaves were analysed for catalase (CAT), glutathione reductase (GR) and superoxide dismutase (SOD) activities. Although a clear effect of $CdCl_2$ on plant growth was observed, the activity of SOD was not altered significantly. However, the CAT activity decreased as the concentration of $CdCl_2$ increased. GR exhibits a significant increase in activity at 2 and 5 mM $CdCl_2$. CAT and SOD isoenzymes were further characterised by analysis in non-denaturing PAGE. Activity staining for SOD revealed up to seven isoenzymes in untreated control and 2 mM $CdCl_2$ treated plants, corresponding to Cu/Zn-SOD isoenzymes. At 5 mM $CdCl_2$, only six Cu/Zn-SOD isoenzymes were observed. No Fe-SOD and Mn-SOD isoenzymes were detected. For CAT, one band of activity was observed.

Additional key words: catalase, glutathione reductase, heavy metals, phytochelatins, reactive oxygen species, *Saccharum officinarum*, superoxide dismutase.

Introduction

In most natural environments, the heavy metal content of the soil is low and does not cause significant phytotoxicity. The increasing contamination and consequent accumulation of heavy metals in the soil can have serious implications on agriculture, since the concentration of such metals can reach unacceptably high contents in plant tissues (Vögeli-Lange and Wagner 1996). Amongst the heavy metals, cadmium (Cd), which is not a nutrient for plants, is particularly toxic and can also accumulate in different human tissues and organs (Yang *et al.* 1997, Thévenod and Friedmann 1999), as well as in several tissues of higher plants (Kovačević *et al.* 1999, Vitória *et al.* 2001).

A group of peptides, which are related to glutathione and termed phytochelatins (PCs) (Leopold *et al.* 1999), can bind Cd and other heavy metals (Grill *et al.* 1987, Rauser 2000), decreasing the concentration of free Cd and impeding direct damage to plant tissues, as well as the production of reactive oxygen species (ROS). The synthesis of these peptides is rapid and the activities of the enzymes involved in this process have been shown to be activated by Cd (Grill *et al.* 1989).

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Abbreviations: BSA - bovine serum albumin; CAT - catalase; DTNB - 5,5'-dithiobis (2-nitrobenzoic acid); DTT - dithiothreitol; EDTA - ethylendiaminetetraacetic acid; GR - glutathione reductase; GSH - reduced glutathione; GSSG - oxidised glutathione; NADPH - nicotinamide adenine dinucleotide phosphate reduced; NBT - nitroblue tetrazolium; PAGE - polyacrylamide gel electrophoresis; PCs - phytochelatins; PVPP - polyvinylpolypyrrolidone; ROS - reactive oxygen species; SDS - sodium dodecyl sulphate; SOD - superoxide dismutase; TEMED - N,N,N',N'-tetramethylethylenediamine.
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Cd has been shown to interfere with chlorophyll biosynthesis and can inhibit enzymes of the photosynthetic Calvin cycle (van Assche and Clijsters 1990). In addition, Cd has been reported to inhibit the activities of several other enzymes involved in nitrogen metabolism (Boussama *et al.* 1999a,b, Kumar and Dubey 1999), glycolysis and the pentose phosphate pathway (Chugh and Sawhney 1999), and sulphate assimilation (Lee and Leustek 1999). Cd can lead to an inhibition of the shoot and root growth (Boussama *et al.* 1999a), accelerated leaf senescence (Siedlecka and Krupa 1999), prevention of stomatal closure, sterility, and rupture of cell membranes (Prasad 1995).

Cd can cause the production of ROS in plants (Foyer *et al.* 1997) due to the formation of superoxide ($O_2^{\bullet-}$) and hydroxyl ($OH^{\bullet}$) radicals (Foyer *et al.* 1994), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Bentivenga *et al.* 1999). Confirmation of ROS production was obtained from the observation that new isoenzymes of peroxidases were formed in plant tissues exposed to Cd (van Assche and Clijsters 1990, Tahlil *et al.* 1999). Other evidence came from the detection of lipid peroxidation and chlorophyll breakdown (Somashekariah *et al.* 1992, Gallego *et al.* 1999).

Peroxide can be metabolised directly by peroxidases, particularly in the cell wall, and by catalase (CAT) in the peroxisome and glyoxisome (Azevedo *et al.* 1998, Polidoros and Scandalios 1999). In the chloroplast, superoxide is converted by superoxide dismutase (SOD) to peroxide which is then detoxified to water and oxygen via the glutathione-ascorbate cycle, which involves the operation of glutathione reductase (GR) (Foyer and Noctor 2000). Three distinct types of SOD enzymes have been detected in plants, which can be classified according to their metal cofactor, Mn, Fe and Cu/Zn. Mn-SOD is located in the mitochondria and peroxisomes, Fe-SOD is associated with the chloroplasts and the abundant Cu/Zn SODs are located in the cytosol, chloroplasts and peroxisomes (Bowler *et al.* 1992, Del Rio *et al.* 1998). The occurrence of SOD activity in such a wide range of metabolic compartments suggests that enzymes of the glutathione-ascorbate cycle, as well as peroxidases, may also play an important role outside the chloroplast (Berna and Bernier 1999).

The effect of Cd on the activities of key enzymes involved in the scavenging of ROS, such as CAT, GR and SOD has only recently been studied in some detail, showing distinct response patterns according to the plant species analysed (Gallego *et al.* 1999, Schickler and Caspi 1999, Ferreira *et al.* 2001, Vitória *et al.* 2001).

In this study, we report the effect of Cd on the activities of the antioxidant enzymes CAT, GR and SOD in the leaves of sugar cane plants. The isolation and classification of SOD isoenzymes has also been determined.

Materials and methods

Plant material: Stem segments of sugar cane (*Saccharum officinarum* L. cv. Copersucar SP80-3280) were pre-treated with fungicide *BENLAT* (0.3 g dm^{-3}) for 15 min and germinated on moistened foam for 5 weeks and then grown in a glasshouse at 28 - 32 °C temperature and a 16-h photoperiod with a irradiance of 300 μmol m^{-2} s^{-1}. A hydroponic system with continuous aeration was set up using pots containing 1.7 dm^3 of Hoagland's nutrient solution. After 4 d, uniform plants were selected and further grown in the same solution, but containing 0, 2, and 5 mM $CdCl_2$ for 96 h. Leaves from each treatment in the periods for 0, 12, 36, 60, 72, 84 and 96 h were collected in liquid nitrogen and stored at -80 °C, prior to extraction.

Enzyme extraction: Extraction and enzyme preparation were carried out at 4 °C. The leaf tissue (5:1 buffer volume:fresh mass) was homogenised in a pestle and mortar with 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 3 mM DTT and 5 % (m/v) insoluble PVPP. The homogenate was centrifuged at 10 000 *g* for 30 min and the supernatant kept stored in separate aliquots at -80 °C to be used for CAT, GR and SOD analysis.

Enzyme assays: CAT activity was determined as described by Barata *et al.* (2000), with some minor modifications. CAT activity was assayed spectrophotometrically at 25 °C in a reaction mixture containing 1 cm^3 100 mM potassium phosphate buffer (pH 7.5), containing 0.025 cm^3 H_2O_2 (30 % solution) prepared immediately before use. The reaction was initiated by the addition of 0.025 cm^3 of plant extract and activity determined by monitoring the degradation of H_2O_2 at 240 nm over 1 min, against a plant extract-free blank. GR activity was determined as described by Chaparro-Giraldo *et al.* (2000) with some minor modifications. GR activity was assayed spectrophotometrically at 30 °C in a mixture consisting of 1 cm^3 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM DTNB, 1 mM GSSG and 0.1 mM NADPH. The reaction was started by the addition of 0.05 cm^3 of plant extract. The rate of reduction of oxidised glutathione was followed by monitoring the increase in absorbance at 412 nm over 1 min.

Polyacrylamide gel electrophoresis (PAGE): Electrophoresis was carried out under non-denaturing condition in 8 and 10 % polyacrylamide gels (16 × 16 cm) for CAT

and SOD activity staining, respectively, as described by Vitória *et al.* (2001). A constant current of 30 mA was applied for 24 h (gels to be stained for CAT activity) or 16 h (gels to be stained for SOD activity) and the temperature maintained at 4 °C. Electrophoresis buffers and gels were prepared as described by Laemmli (1970) except that SDS was excluded. Equal amounts of protein were loaded on to each lane.

Enzymes activity staining: CAT activity in non-denaturing PAGE gels was determined as described by Woodbury *et al.* (1971). Gels were incubated in 0.003 % H_2O_2 for 10 min and developed in a 1 % (m/v) $FeCl_3$ and 1 % $K_3Fe(CN_6)$ (m/v) solution for 10 min. 1 unit of bovine liver CAT (*Sigma*, St. Louis, USA) was applied to all gels to serve as a positive control of CAT activity. SOD activity was determined as described by Beauchamp and Fridovich (1971) and modified by Azevedo *et al.* (1998). The gels were rinsed in distilled-deionized water and incubated in the dark for 30 min at room temperature in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.8), 1 mM EDTA, 0.05 mM riboflavin, 0.1 mM NBT and 0.3 % (v/v) TEMED. The gels were then rinsed with 300 cm^3 (3 × 100 cm^3) distilled-deionized water and illuminated in water until the development of colourless bands of SOD activity in a purple-stained gel was visible. The reaction was stopped by transferring the gel to a 7 % (v/v) acetic acid solution. 1 unit of bovine liver SOD (*Sigma*) was applied to all gels to serve as a positive control of SOD activity. SOD isoenzymes were classified by testing leaf samples, as described previously by Azevedo *et al.* (1998) using KCN and H_2O_2 as inhibitors. Mn-SOD isoenzymes are resistant to both inhibitors, Fe-SOD isoenzymes are resistant to KCN and inhibited by H_2O_2, and Cu/Zn-SOD isoenzymes are inhibited by both inhibitors.

Determination of protein concentration: The total soluble protein concentration in all extracts was determined by the method of Bradford (1976) using BSA (*Sigma*) as a standard.

Statistical analysis: Three repetitions per treatment were made, and enzyme activities were assayed three times. Analysis of variance (*ANOVA*) and regression analysis were made of the data obtained.

Results

Sugar cane plants were initially grown in $CdCl_2$ concentrations of 0.1 and 1 mM for a 15-d period in Hoagland's nutrient solution. At the end of the period, no alteration in growth was observed at 0.1 mM $CdCl_2$ concentration, whereas at 1 mM a small growth inhibition was observed (data not shown). Based on these preliminary results, a second experiment using 2 and 5 mM $CdCl_2$ was carried out. Both concentrations inhibited growth, leading to severe damage of the leaves after 96 h of treatment.

In relation to the response of antioxidant enzymes to Cd treatment, statistical analyses of CAT specific activity (Table 1) showed highly significant differences among the tested treatments, and none significant differences between the additional control treatment. The factorial design analysis showed high significance for the time effect, dosage effect and for the interaction between time of exposure and different dosage (Table 1). In plants that were not exposed to $CdCl_2$, there was an immediate increase in CAT activity after 12 h, which was followed by a decrease at 36 h and a restoration of activity at 60 and 72 h (Fig. 1*A*). Following this time there was a further decrease in CAT activity at 84 h and a dramatic increase at 96 h. In the plants treated with 2 mM $CdCl_2$, there was a 50 % loss of CAT activity after 12 h of exposure, but following this, the CAT activity followed

Table 1. Catalase (CAT) and glutathione reductase (GR) activities. Statistical analysis. SM and probability × 10^{-5}. General means for CAT and GR = 5.439 and 1.015, respectively. Variation coefficient for CAT and GR = 6.962 and 12.390 %, respectively.

Sources of variation	DF		SM		Probability > F	
	CAT	GR	CAT	GR	CAT	GR
Treatments	18	18	80.60	5.39	10	4
Control *vs* factorial	1	1	41.50	0.083	78 200	78 200
Time of Cd exposure	5	5	202.70	1.72	1	100
Cd dosage	2	2	46.30	31.30	30	1
Time dosage	10	10	80.10	4.58	1	23
Error	36	35	3.97	1.00		
Total	54	53				

similar pattern as the untreated controls. In the plants treated with 5 mM $CdCl_2$, there was a slight reduction in CAT activity as compared to the 2 mM treated plants, which decreased further after 72 h, but this was again followed by a dramatic increase until 96 h (Fig. 1*A*).

CAT activity was also measured on non-denaturing PAGE. One major band of CAT activity was observed following electrophoresis of leaf extracts (Fig. 2). The band of CAT activity, exhibited a slight widening in the extracts of the 2 mM $CdCl_2$ treated plants after 72 h, but there was little effect on total activity (Fig. 2). In the 5 mM $CdCl_2$ treated plants, there was a dramatic loss of CAT activity between 12 and 36 h, to 10 % of the initial activity. The activity fell to almost zero after 96 h (Fig. 2).

Statistical analysis of GR specific activity (Table 1) also showed highly significant differences among the tested treatments, and no significant differences between the additional control treatment and the factorial design. The factorial design analysis showed high significance for time effect, for dosage effect, and significance for the interaction between treatment time and dosage (Table 1).

During the first 36 h of treatment, there was no significant effect of the $CdCl_2$ treatment at either 2 mM or 5 mM on GR activity (Fig. 1*B*). However after 60 h, the activity of GR was considerably reduced in the untreated control plants, while in both 2 mM and 5 mM

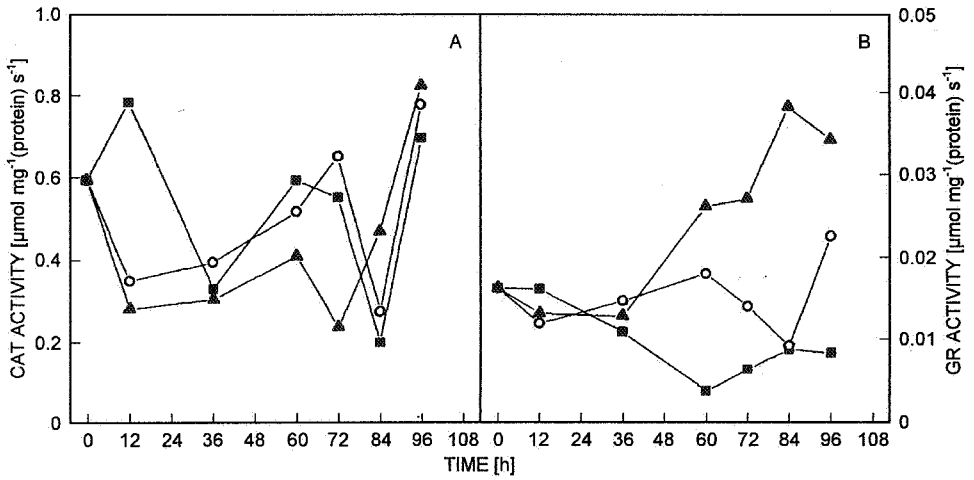

Fig. 1. The specific activities of catalase (CAT) (*A*) and glutathione reductase (GR) (*B*) in the leaves of sugar cane seedlings during 96-h treatment. Control (*squares*), 2 mM $CdCl_2$ (*circles*) and 5 mM $CdCl_2$ (*triangles*).

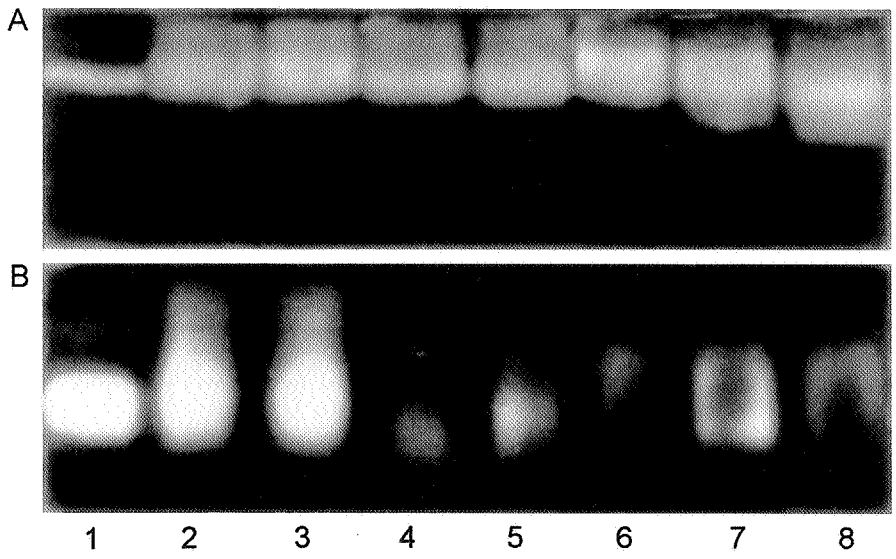

Fig. 2. Activity staining for catalase (CAT) in sugar cane leaves grown for a 96 h period in 2 mM (*A*) and 5 mM $CdCl_2$ (*B*). *Lane 1* - CAT standard from bovine liver; *lane 2* - control - 0 h; *lane 3* - 12 h; *lane 4* - 36 h; *lane 5* - 60 h; *lane 6* - 72 h; *lane 7* - 84 h; *lane 8* - 96 h. 60 µg of protein from leaves were loaded onto each gel lane.

$CdCl_2$ treatments there was an increase in GR activity. Although the GR activity decreased in the 2 mM $CdCl_2$ treated plants after 84 h, the final activity at 96 h was almost 3-fold higher than in the untreated control plants. In the 5 mM $CdCl_2$ treated plants, the GR activity increased steadly between 36 and 96 h, with the final activity being 5-fold higher than in the untreated control plants (Fig. 1*B*).

SOD activity staining in non-denaturing PAGE revealed several clear bands in all experiments (Fig. 3). In the leaf tissue tested, SOD activity did not show any great variation in terms of total SOD activity, except for one specific SOD isoenzyme at the highest $CdCl_2$ concentration (Fig. 3*C*). Furthermore, seven SOD isoenzymes were identified in the leaves of the control and $CdCl_2$ treated plants. The distinct bands were classified according to the inhibition caused by hydrogen peroxide (H_2O_2) and KCN (data not shown). All SOD isoenzymes were classified as Cu/Zn-SOD (inactivated by both inhibitors). Fe-SOD and Mn-SOD isoenzymes were not observed in leaves of sugar cane. The classification of the isoenzymes was carried out using a tobacco leaf SOD extract as a control (Azevedo *et al.* 1998, Barata *et al.* 2000).

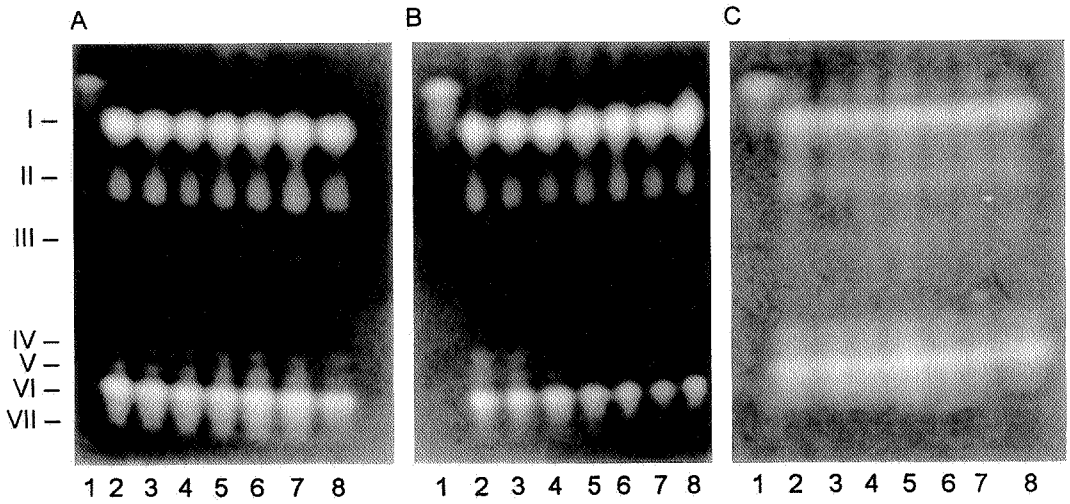

Fig. 3. Activity staining for superoxide dismutase (SOD) in sugar cane leaves grown for a 96 h period in control (*A*), 2 mM (*B*) and 5 mM $CdCl_2$ (*C*). *Lane 1* - SOD standard from bovine liver; *lane 2* - without $CdCl_2$ - 0 h; *lane 3* - 12 h; *lane 4* - 36 h; *lane 5* - 60 h; *lane 6* - 72 h; *lane 7* - 84 h; *lane 8* - 96 h. 100 μg of protein from leaves were loaded onto each gel lane.

Discussion

CAT has been shown to be present in distinct isoforms in plants (Kendall *et al.* 1983, Willekens *et al.* 1994, Polidoros and Scandalios 1999). In sugar cane, only one band of CAT activity was observed. CAT activity was measured by two distinct methods, and up until 72 h there was clear evidence that 5 mM $CdCl_2$ had a far greater inhibitory effect on CAT activity than 2 mM $CdCl_2$, irrespective of the enzyme assay used. However, in the 84-h and 96-h time periods, there was evidence of recovery of enzyme activity in all treatments, when the spectrophotometer assay was employed. This recovery was not clearly observed in the gel assay of extracts from plants that had been exposed to 5 mM $CdCl_2$, possibly due to interference with the ferric chloride/ferricyanide assay mechanism. The results observed for CAT could indicate a dose-dependent response and that at high concentrations of Cd, the antioxidant enzyme capacity is decreased. In previous reports, CAT activity from *Phaseolus aureus* (Shaw 1995), *Phaseolus vulgaris* (Chaoui *et al.* 1997), *Pisum sativum* (Dalurzo *et al.* 1997), *Lemna minor* (Mohan and Hossetti 1997), *Helianthus annuus* (Gallego *et al.* 1999) and *Amaranthus lividus* (Bhattacharjee 1998), has been shown to decline when exposed to Cd. On the other hand, in *Agropyron repens* (Brej 1998) and radish (Vitória *et al.* 2001), the activity was shown to increase in leaves and roots in response to Cd and remain unaltered in soybean leaves (Ferreira *et al.* 2001).

Since SOD dismutates ROS into hydrogen peroxide, the results obtained suggest that, although Cd may generate an oxidative stress, the SOD activity is sufficient to cope with an increased concentration of such radicals. In a similar manner to CAT, the results reported in the literature also indicate distinct responses depending on plant species and the length of Cd treatment. With the exception of pea (Dalurzo *et al.* 1997) and *Alyssum* species (Schickler and Caspi 1999), which have shown an increased SOD activity in response to Cd treatment, the

majority of the other plant species tested exhibited no changes or a decrease in total SOD activity (Gallego *et al.* 1996, Bhattacharjee 1998, Gallego *et al.* 1999, Ferreira *et al.* 2001). An increase in SOD activity has also been recently reported in radish in response to Cd treatment, however, such an increase was not correlated with any specific SOD isoenzyme (Vitória *et al.* 2001).

There is considerable evidence that GSH is used to form PCs in higher plants (Leopold *et al.* 1999). The increase in GR activity detected in the leaves of sugar cane suggests that this enzyme is responding to Cd stress by maintaining glutathione in the reduced form prior to incorporation into PCs. Such a possibility has recently been suggested for the effect of Cd in radish tissues (Vitória *et al.* 2001) and soybean roots (Ferreira *et al.* 2001), whereas in sunflower GSH was diminished when grown in the presence of $CdCl_2$ (Gallego *et al.* 1999). GR activity has been shown to increase in response to Cd treatment in *Phaseolus vulgaris* and potato, however, such an increase was dependent upon Cd concentration and the time of exposure (Chaoui *et al.* 1997, Stroinski *et al.* 1999). On the other hand, leaves of transformed poplar overexpressing γ-glutamylcysteine synthetase exhibited GR activity unchanged by exposure to Cd (Arisi *et al.* 2000). A second possible reason for the increase in GR activity could be the operation of the glutathione-ascorbate cycle in detoxifying the ROS induced by Cd (Ferreira *et al.* 2001, Vitória *et al.* 2001).

In leaves, CAT is essential for the metabolism of the peroxide produced in the peroxisome (Kendall *et al.* 1983), whereas SOD isoforms are directly related to the scavenging of ROS in different organelles. Cd treatment of sugar cane plants has caused a response by stimulating an increase in GR activity in the leaves, suggesting two possible situations, one in which involves the formation of PCs at a higher rates, the other the detoxification of ROS. The second possibility is less likely, as the SOD isoenzymes did not respond directly to the $CdCl_2$ treatment, at least at the concentrations tested and during the period of treatment.

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