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Photochemical damage and comparative performance of superoxide dismutase and ascorbate peroxidase in sugarcane leaves exposed to paraquat-induced oxidative stress

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Abstract

The physiological responses of sugarcane (*Saccharum officinarum* L.) to oxidative stress induced by methyl viologen (paraquat) were examined with respect to photochemical activity, chlorophyll content, lipid peroxidation and superoxide dismutase (SOD) and ascorbate peroxidase (APX) activities. Thirty-day-old sugarcane plants were sprayed with 0, 2, 4, 6 and 8 mM methyl viologen (MV). Chlorophyll fluorescence was measured after 18 h and biochemical analyses were performed after 24 and 48 h. Concentrations of MV above 2 mM caused significant damage to photosystem II (PSII) activity. Potential and effective quantum efficiency of PSII and apparent electron transport rate were greatly reduced or practically abolished. Both chlorophyll and soluble protein contents steadily decreased with MV concentrations above 2 mM after 24 h of exposure, which became more pronounced after 48 h, achieving a 3-fold decrease. Insoluble protein contents were little affected by MV. Oxidative stress induced by MV was evidenced by increases in lipid peroxidation. Specific activity of SOD increased, even after 48 h of exposure to the highest concentrations of MV, but total activity on a fresh weight basis did not change significantly. Nondenaturing PAGE assayed with H_2O_2 and KCN showed that treatment with MV did not change Cu/Zn-SOD and Mn-SOD isoform activities. In contrast, APX specific activity increased at 2 mM MV but then dropped at higher doses. Oxidative damage induced by MV was inversely related to APX activity. It is suggested that the major MV-induced oxidative damages in sugarcane leaves were related to excess H_2O_2, probably in chloroplasts, caused by an imbalance between SOD and APX activities, in which APX was a limiting step. Reduced photochemical activity allowed the early detection of the ensuing oxidative stress.
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1. Introduction

The active component of the herbicide paraquat, methyl viologen (MV; 1,1′-dimethyl-4,4′-bipyridinium dichloride), exerts its phytotoxic effects on plants by transferring electrons from photosystem I to molecular oxygen, resulting in an accumulation of superoxide radicals in chloroplasts

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[1]. Thus, MV toxicity results from deleterious reactions of cellular components with reactive oxygen species (ROS)[1] such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH.). ROS can trigger harmful reactions such as DNA alterations, protein degradation and lipid peroxidation, affecting key components of plant cell metabolism [2,3]. It has been reported that paraquat can readily penetrate the leaf cuticle, quickly decreasing photosynthetic activity, rupturing membranes and causing loss of turgor in treated leaves within hours [4,5]. In advanced stages of phytotoxicity, necrotic lesions appear on the leaf surface and the plant finally dies.

Resistance to environmental stress is a major selection criterion in plant breeding. Plant cellular and molecular tolerance mechanisms to different types of stresses have been intensively studied. Plant species have evolved a complex antioxidant system to prevent the harmful effects of ROS, which play a major role in stress tolerance [6,7]. Oxidative damage ensues when this complex system fails to limit ROS accumulation. This defense system involves lipid-soluble antioxidants (α-tocopherol and carotenoids), water-soluble reductants (glutathione and ascorbate) and enzymes such as catalase (CAT), glutathione reductase (GR), ascorbate peroxidase (APX) and superoxide dismutase (SOD) [8]. Among the antioxidative enzymes, SOD plays an essential role in the protection of cellular components against oxidative damage [9,10], converting O_2^- to H_2O_2. The hydrogen peroxide is then eliminated by H_2O_2-scavenging enzymes such as peroxidases and catalases. APX is widely distributed in the cytosol and other organelles [11] and is very effective in scavenging H_2O_2 generated by SOD through the Halliwell-Asada pathway, especially in chloroplasts where CAT is virtually absent [12]. However, APX can be inactivated by excess H_2O_2 [13,14].

In higher plants, SOD is classified according to its respective metal cofactor: Cu/Zn-SOD, Fe-SOD and Mn-SOD. The Cu/Zn-SOD and Mn-SOD have been identified in maize and other plant species [15–17]. The Cu/Zn-SOD is present in chloroplasts or in the cytosol, while the Mn-SOD is located in mitochondria [18]. The Fe-SOD is also present in chloroplasts [19].

In chloroplasts under normal physiological conditions, SOD and APX activities are closely balanced, i.e. the excess of superoxide radicals is eliminated by SOD, generating H_2O_2 that is scavenged through APX activity. However, under stressful oxidative conditions, like those due to excess superoxide radicals generated by exposure to MV, SOD or APX activities can become limiting. Adding to the problem, antioxidant enzymes themselves (as well as other proteins) can be damaged by the unchecked surge of ROS. Some experimental evidence has suggested that APX is more labile than SOD under oxidative stress conditions generated by methyl viologen in plants like spinach [13], transgenic tobacco [14] and wheat [20].

In the present work we investigated the effects of short-term, acute exposure to paraquat in sugarcane leaves by comparing photochemical activity, oxidative damage and SOD and APX activities. We were interested in examining the hypothesis that APX activity is a limiting step in ROS detoxification when compared to SOD activity.

2. Materials and methods

2.1. Plant material, growth conditions and treatments

Sugarcane (*Saccharum officinarum* L.) plants of a paraquat-resistant cultivar, SP 80-3280, were reproduced from stem segments containing a single bud supplied by the Centro de Tecnologia Canavieira (CTC), Piracicaba, São Paulo, Brazil. The plants were grown in 3-L pots containing commercial substrate (Plantmax®, Eucatex Inc.) under greenhouse conditions, viz. average maximum and minimum air temperature of 29.1 and 23.2 °C, respectively, average relative humidity of 76%, maximum photosynthetic photon flux density (PPFD) of approximately 750 μmol m^{-2} s^{-1} and a 12 h photoperiod. Thirty days after germination, different concentrations of paraquat (0, 2, 4, 6 and 8 mM of methyl viologen) were sprayed until all leaves were completely wetted.

2.2. Measurements of chlorophyll fluorescence

Chlorophyll fluorescence was measured after 18 h of exposure to MV with a modulated fluorometer model PAM-2000 (Walz, Germany). Measurements of minimum (F_o) and maximum (F_m) chlorophyll fluorescence were taken in dark-adapted tissues (30 min) using Walz leafclips. In light-adapted samples (PPFD of 325 μmol m^{-2} s^{-1} at room temperature), steady-state (F) and maximum (F'_m) chlorophyll fluorescence were also evaluated by attaching the optic fiber to a leafclip holder, which regulates the angle (60°) and the distance (0.5 cm) between the optic fiber and leaf sample. F_m and F'_m were assessed after a light saturation pulse (15.000 μmol m^{-2} s^{-1}) in both dark and light conditions. These data were used to calculate variable chlorophyll fluorescence in both dark ($F_v = F_m - F_o$) and light-adapted ($\Delta F = F'_m - F$) states, from which other important photochemical variables were estimated: potential (F_v/F_m) and effective ($\Delta F/F'_m$) quantum efficiency of photosystem II (PSII); apparent electron transport rate (ETR); and relative excess energy at the PSII level (EXC) [21–24]. ETR was calculated as $ETR = (\Delta F/F'_m \times PPFD \times 0.5 \times 0.84)$,

[1] *Abbreviations:* APX, ascorbate peroxidase; CAT, catalase; DHAR, dehydroascorbate reductase; ETR, apparent electron transport rate; EXC, relative energy excess at PSII level; F_o, minimum chlorophyll fluorescence; F_m, maximum chlorophyll fluorescence; F_v/F_m, potential quantum efficiency of PSII; GR, glutathione reductase; MV, methyl viologen; PAGE, polyacrylamide gel electrophoresis; PPFD, photosynthetic photon flux density; PSII, photosystem II; PVPP, polyvinylpolypirrolidone; ROS, reactive oxygen species; SOD, superoxide dismutase; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive species; $\Delta F/F'_m$, effective quantum efficiency of PSII.

where 0.5 is the presumed fraction of the excitation energy distributed to PSII and 0.84 is the assumed fraction of light absorbed by the leaf. EXC was calculated according to Bilger et al. [21] as $EXC = [(F_v/F_m) - (\Delta F/F'_m)]/(F_v/F_m)$.

All photochemical measurements were performed on the +3 fully expanded leaf.

2.3. Biochemical analyses

Biochemical determinations were performed in mixed samples from all sugarcane leaves collected after 24 and 48 h of exposure to paraquat. Immediately after harvest, leaf tissues were frozen in liquid-nitrogen and stored at −80 °C prior to analyses.

2.3.1. Determination of leaf chlorophyll content

Chlorophyll was extracted with acetone in a mortar, using a proportion of 500 mg of fresh leaf tissue to 5 mL of acetone (80%, v/v). Chlorophyll concentration was measured by the classical Arnon [25] method. After centrifugation (10 min at 5000*g*), the absorbance of the supernatant was measured at 663 and 645 nm. To quantify total chlorophyll contents (mg g^{-1} FW), the following equation was used: total chlorophyll $= (20.2 \times A645 + 8.02 \times A663) \times V/1000W$, where, A = absorbance; V = final volume of the extract and W = fresh weight in grams [25].

2.3.2. Lipid peroxidation

Lipid peroxidation was evaluated in terms of the concentration of thiobarbituric acid-reactive substances (TBARS) as described in [26] with minor modifications. One gram of fresh leaf was homogenized in 3 mL of 1.0% (w/v) TCA at 4 °C. The homogenate was centrifuged at 20,000*g* for 15 min and 0.5 mL of the supernatant was added to 3 mL of 0.5% (v/v) TBA in 20% TCA. The mixture was incubated at 95 °C in a shaking water bath for 50 min, and the reaction stopped by cooling the tubes in an ice water bath. Samples were then centrifuged at 9000*g* (for 10 min), and the absorbance of the supernatant was read at 532 nm. The value for nonspecific absorption at 660 nm was subtracted from readings at 532 nm. The concentration of TBARS was calculated using the absorption coefficient of 155 mM^{-1} cm^{-1}.

2.3.3. Determination of soluble and insoluble protein concentration

One gram of fresh leaves was macerated in a mortar with 2 mL of acetone (80%, v/v) to remove pigments. After centrifugation at 6000*g* for 10 min, the pellet was mixed by agitation for 1 h with Tris buffer (40 mM, pH 7.8), containing 350 mM manitol, 2 mM EDTA and PVPP 3% (w/v). Following centrifugation (10,000*g* for 20 min at 4 °C), the supernatant was collected as the soluble protein fraction. The pellet was agitated for 1 h with 2 mL of lyse solution (8 M urea, 1 M thiourea, 2% CHAPS and 50 mM DTT). After another centrifugation (10,000*g* for 20 min at 4 °C), the supernatant, i.e. the insoluble protein fraction, was kept at −80 °C for posterior analysis. Protein concentration was determined according to the Bradford [27] method using bovine serum albumin as standard.

2.3.4. Enzyme extraction and activity assays

2.3.4.1. Extraction. Sugarcane leaves (1.0 g fresh weight) were homogenized for 5 min with a mortar and pestle in 1.5 mL of ice-cold 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 1 mM ascorbic acid. After filtration with cheesecloth, the homogenate was centrifuged at 13,000*g* for 20 min and the supernatant (crude extract) was used as the source of enzymes. All steps were carried out at 0–4 °C. Protein contents of the crude extracts were determined using the Bradford [27] protein assay, with bovine serum albumin as a standard.

2.3.4.2. SOD activity. The activity of superoxide dismutase (EC 1.15.1.1) was determined by adding 50 μL of the crude enzyme extract to a solution containing 13 mM L-methionine, 75 μM *p*-nitro blue tetrazolium chloride (NBT), 100 μM EDTA and 2 μM riboflavin in a 50 mM potassium phosphate buffer (pH 7.8). The reaction took place in assay tubes upon illumination (30 W fluorescent lamp at 25 °C) for 15 min [12]. The blue formazane produced by NBT photoreduction was measured by the absorbance at 620 nm in a spectrophotometer. The control reaction mixture had no enzyme extract. The blank solution had the same complete reaction mixture but was kept in the dark. One SOD unit of activity was defined as the amount of enzyme required to inhibit 50% of NBT photoreduction in comparison to tubes lacking the plant extract. Activity was expressed as units per mg soluble protein per min (UA mg^{-1} protein min^{-1}).

2.3.4.3. APX activity. The activity of ascorbate peroxidase (EC 1.11.1.11) was determined by adding 100 μL of the crude enzyme extract to 0.5 mL of a solution containing 0.5 mM L-ascorbic acid diluted in 50 mM potassium phosphate buffer (pH 6.0) and 2 mM H_2O_2. The decrease of absorbance at 290 nm was monitored during 120 s [28] modified by Koshiba [29]. One APX unit of activity was considered as a 0.1 decrease in absorbance units that occurred after 1 min of reaction. Enzyme activity was expressed as UA mg^{-1} protein min^{-1}. All determinations were assayed in triplicates.

2.3.4.4. Activity of SOD isoforms in native electrophoresis. Gel activity assays were performed on samples containing equal amounts of protein (40 μg) and separated by nondenaturing 12.5% PAGE at 4 °C. The gels were placed in a solution of 0.05 M potassium phosphate (pH 7.8), containing 1 mM EDTA, 0.1 mM nitroblue tetrazolium and 0.05 mM riboflavin, for 15 min. For identification of Mn-SOD and Cu/Zn-SOD activities, the gels were immersed in 5 mM H_2O_2 or 2 mM KCN in 0.1 M potassium phosphate (pH 7.8), respectively, for 15 min in the dark. Both H_2O_2 and KCN are inhibitors of Cu/Zn-SOD whereas

H_2O_2 is an inhibitor (but KCN is not) of Fe-SOD. Both H_2O_2 and KCN do not inhibit Mn-SOD. Afterwards, all gels were exposed to illumination by a 30 W fluorescent lamp at 25 °C until the appearance of the bands [18].

2.3.5. Statistical analysis

The experiment was arranged in a completely randomized design, with four independent replicates, each consisting of one pot containing one individual plant. Data were analyzed by ANOVA and means were compared by the Tukey test at the 0.05 level of confidence. The standard deviation was plotted in all graphs.

3. Results

The chlorophyll fluorescence data (Fig. 1A–C) clearly show that paraquat, at concentrations of methyl viologen (MV) above 2 mM, severely damaged the photochemistry of sugarcane plants after 18 h. Maximum chlorophyll fluorescence (F_m), potential (F_v/F_m) and effective ($\Delta F/F'_m$) quantum efficiency of photosystem II, and apparent electron transport rate (ETR), which are variables related to photochemical activity, all decreased abruptly at concentrations higher than 2 mM of MV after 18 h of exposure (Fig. 1A–C, respectively). In contrast, minimum chlorophyll fluorescence (F_o) increased slightly under 4, 6 and 8 mM as compared to 0 and 2 mM (Fig. 1A). As a consequence of photochemical damage, there was a prominent increase in relative excess energy (EXC) at the photosystem level following exposure to MV at concentrations higher than 2 mM (Fig. 1C).

The effects of MV on chlorophyll breakdown were somewhat different from those related to photosystem II efficiency, with an apparent lag period between these events. Even after 24 h of exposure, total chlorophyll contents were similar to those of the controls (Fig. 2A), with the exception of the 2 mM MV treatment which displayed a slight increase. However, chlorophyll content was significantly reduced by MV concentrations higher than 2 mM after 48 h exposure. The paraquat-treated sugarcane leaves showed a progressive increase in lipid peroxidation, as indicated by the level of TBARS formation, especially in MV concentrations higher than 2 mM (Fig. 2B). This oxidative effect of MV was more pronounced after 48 h of treatment, with TBARS levels being more than 2-fold higher relative to untreated plants.

Exposure to paraquat caused a progressive reduction in soluble protein concentration. After 24 h of treatment, protein concentration decreased slightly at MV concentrations higher than 2 mM (Fig. 2C). This decrease was prominent at all concentrations after 48 h, reaching values 3-fold lower than those of controls, at concentrations 4 mM or higher (Fig. 2C). The insoluble protein concentration did not change significantly by MV treatment (Fig. 2D). Interestingly, leaf SOD specific activity, expressed in terms of soluble protein, increased significantly with increasing doses of MV, reaching a 3-fold increment after 48 h (Fig. 2E). This increase was due to decreased soluble protein contents. However, even after 48 h of exposure, when the leaves were severely injured, total SOD activity, if expressed on a fresh weight basis, remained relatively constant (Fig. 2C and E).

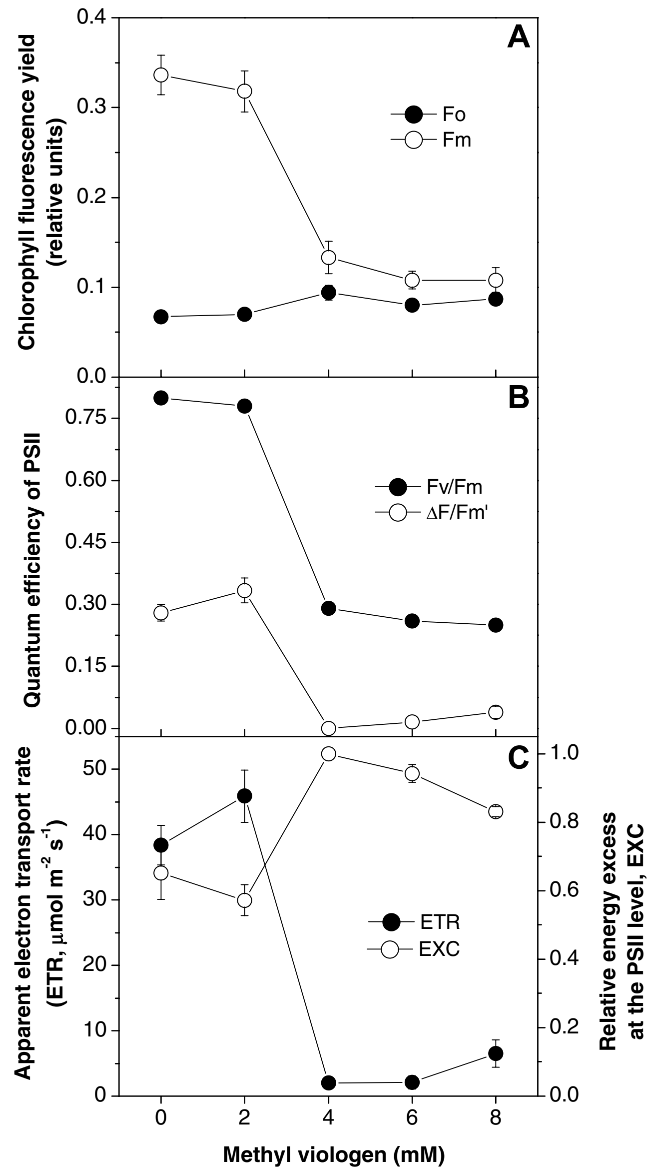

Fig. 1. Chlorophyll fluorescence variables in sugarcane leaves as affected by methyl viologen after 18 h of exposure. Minimum (F_o) and maximum (F_m) chlorophyll fluorescence (A); potential (F_v/F_m) and effective ($\Delta F/F'_m$) quantum efficiency of photosystem II (B); relative energy excess at PSII level, EXC (C) and apparent electron transport rate, ETR (C). Each symbol represents the mean value of four replicates ± SD.

Three distinct SOD isoenzyme bands (Fig. 3) were observed by nondenaturing PAGE of proteins extracted from the paraquat-treated sugarcane leaves. The highest molecular mass band corresponds to the Mn-SOD isoform according to its inhibition assay against H_2O_2 and KCN (this isoform was insensitive to both inhibitors). The other two isoforms, presenting similar molecular mass, were identified as Cu/Zn-SOD based on their sensitivity to

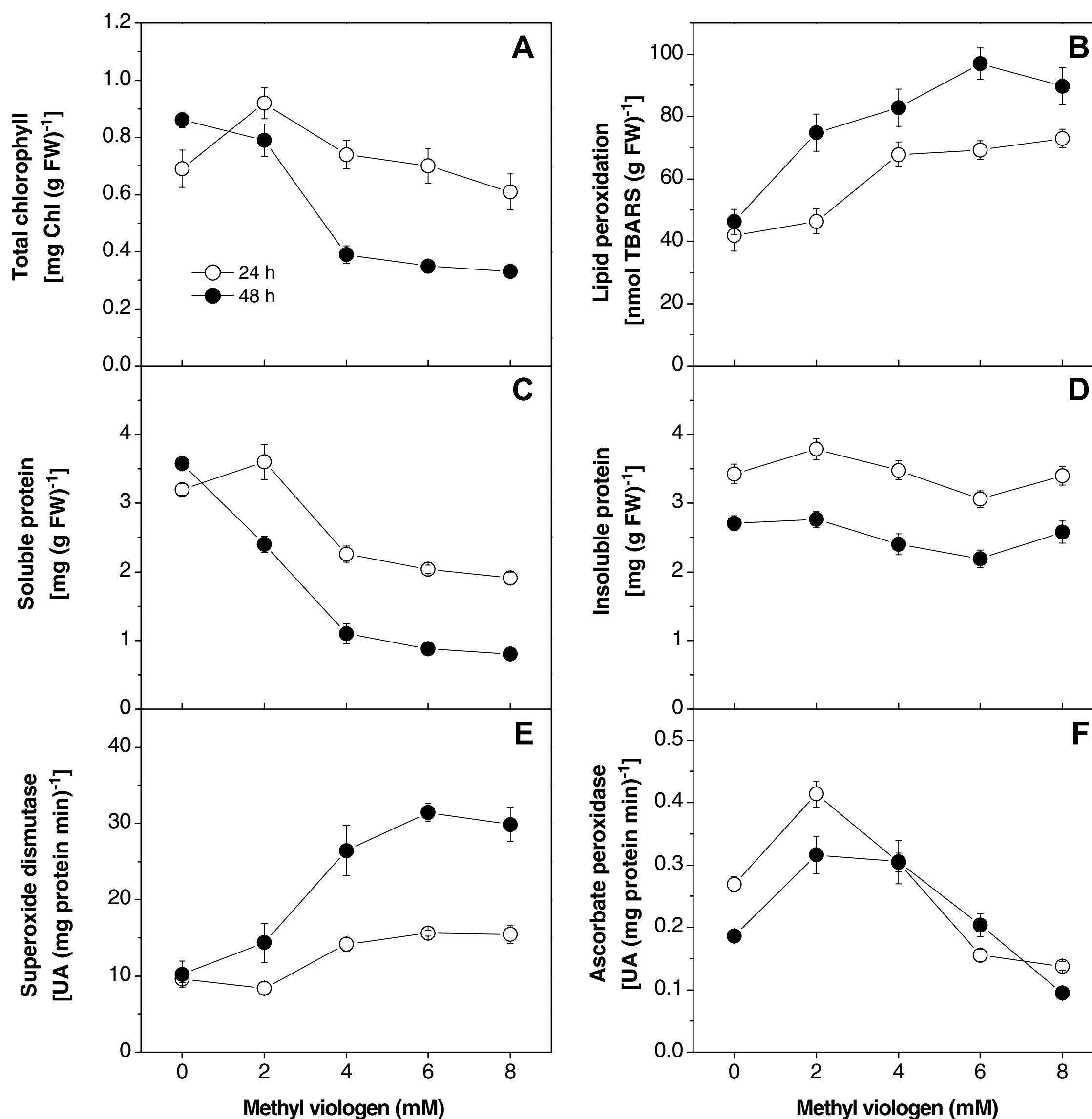

Fig. 2. Total chlorophyll content (A), lipid peroxidation (B), soluble (C) and insoluble (D) protein contents, superoxide dismutase (E) and ascorbate peroxidase (F) activities in leaves of sugarcane submitted to methyl viologen application. Samples were collected after 24 and 48 h of exposure. Each symbol represents the mean value of four replicates ± SD.

KCN and H_2O_2. As shown in Fig. 3, there was no clear difference among treatments after 24 h of exposure to MV. Similarly, no isoform was significantly affected in plants even after 48 h of exposure, when the leaves were severely injured. Although no increase in specific activity of any of the SOD isoforms was observed by native PAGE electrophoresis (Fig. 3), in contrast to total activity (Fig. 2E), the results do indicate that these three isoforms were very resistant to oxidative stress induced by MV.

In contrast to SOD, leaf APX specific activity was only stimulated by 2 mM of MV after 24 h and by 2 and 4 mM MV after 48 h of treatment (Fig. 2F). Above these concentrations, APX specific activity decreased steadily and in parallel to decreases in the soluble protein concentration (Fig. 2C and F). Therefore, total APX activity, on a fresh weight basis, was stimulated only at 2 mM MV at 24 h, but dropped at higher concentrations of MV. The toxic effect of MV on total APX activity was even more pronounced after 48 h of treatment.

Taken together, these results demonstrate that paraquat initially and more severely affects photosystem II activity (Fig. 1), followed by lipid peroxidation (Fig. 2B) and APX activity (Fig. 2F) in sugarcane.

4. Discussion

In this study, treatment of sugarcane plants with methyl viologen (paraquat herbicide) resulted in photochemical damage only at concentrations of 4 mM and above, as early as 18 h after treatment. However, after 24 h of treatment with MV, total chlorophyll content was not significantly altered compared to control, even at the highest

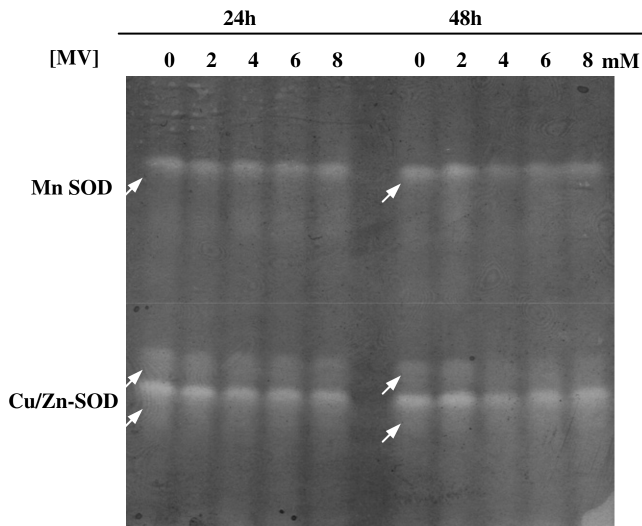

Fig. 3. Superoxide dismutase isoform activities by nondenaturing 12.5% PAGE from sugarcane leaves exposed to methyl viologen (MV) for 24 and 48 h. The bands corresponding to Mn-SOD and Cu/Zn-SOD activities (arrows) were identified after gel treatment with KCN and H_2O_2 inhibitors. The Mn-SOD isoform was insensitive to both inhibitors whereas the Cu/Zn-SOD activity was inhibited for both inhibitors.

level of MV (8 mM). At a concentration of 2 mM MV, physiological alterations were observed only after 48 h with respect to lipid peroxidation and soluble protein content. These results corroborate the view that SP 80-3280 is a paraquat-resistant sugarcane genotype since 4 mM MV is a very toxic level for most crops [3].

It is known that environmental stresses generally induce an increase in F_o and a decrease in F_m [23,30]. Increases in F_o are related to damages in thylakoid membrane structure with partial dissociation of LHCII from PSII [23,30]. Reduction in F_m is related to inhibition of photosynthetic activity due to dysfunction in PSII activity [23]. The latter was more evident in this study, where oxidative damage was induced by MV (Fig. 1A).

Variations in F_o but particularly in F_m caused significant decreases in F_v/F_m (~0.25–0.30) at MV doses higher than 2 mM (Fig. 1B). These values are indicative of pronounced photoinhibition of photosynthesis [31], despite the low light intensity applied (325 μmol m^{-2} s^{-1}). Indeed, excess energy can occur under low PPFD when there are decreases in CO_2 fixation and consequently a reduction in the ATP and NADPH consumed. This excess light energy was indicated by increases in EXC under MV concentrations higher than 2 mM (Fig. 1C). Under light-energy pressure (light-adapted state), photochemical reactions also showed impairments caused by application of MV (Fig. 1B and C). Since ETR may be considered an overall index of photochemical activity, the data suggest that oxidative damage practically de-activated the electron transport chain in thylakoid membranes when plants were treated with MV concentrations higher than 2 mM (Fig. 1C). An important aspect is that the above changes in photochemical variables (Fig. 1) were observed prior to the development of visual symptoms and biochemical responses (Figs. 2 and 3), which suggests that the measurement of chlorophyll fluorescence is a potential tool to detect early stages of oxidative damage.

Methyl viologen acts by blocking PSI electron flux, hindering energy transfer from chlorophyll to the P680 reaction center and consequently originating a very reactive molecule [32], triplet chlorophyll (^{3}Chl). The high concentrations of reduced photosynthetic electron carriers favor a one-electron transfer reaction to oxygen producing the superoxide anion radical ($O_2^{\cdot -}$) from which other ROS are derived. However, whether oxidative stress and damage to cellular components will ensue depends on the extent to which these ROS are neutralized by the cellular antioxidant system. In this study, oxidative stress was clearly established, as indicated by lipid peroxidation (Fig. 2B). In addition, the intensity of lipid peroxidation was higher in plants exposed to MV for 48 h when compared to 24 h, suggesting a time-dependent development of oxidative damage in cell membranes. As reported in [12], we also argue that TBARS production is a good indicator for evaluating oxidative damage in plant tissues.

SOD activity, expressed on a soluble protein basis, was stimulated in MV concentrations higher than 2 mM after both 24 and 48 h exposure (Fig. 2E), but was stable when expressed on a fresh weight basis (data not shown). Specific analyses for Mn-SOD (H_2O_2 and KCN-resistant) and Cu–Zn-SOD (H_2O_2 and KCN-sensitive) showed that the activities of these SOD isoforms did not change significantly with doses of MV. It is unclear why the intensity of the bands in the native PAGE (Fig. 3) did not increase as did total SOD activity (Fig. 2E). This may have been due to the additive effects of two factors: (a) The detection of activity in the bands is less sensitive than the detection in solution and (b) Several SOD isoforms exist in the leaf tissue [18]. These different isoforms may not have all been adequately detected in the gels. Nonetheless, the results in both Figs. 2E and 3 show that SOD activity was resistant to the imposed oxidative stress.

In contrast to SOD, leaf APX activity, as expressed on both a soluble protein and a fresh weight basis, was stimulated only by 2 mM of MV after 24 and 48 h exposure and dropped abruptly at MV 4 mM and higher (Fig. 2F). Thus, the correlative data presented here strongly suggest that APX activity, when compared to SOD activity, was a limiting step to the neutralization of ROS generated by MV exposure.

In plant chloroplasts, to avoid oxidative stress, SOD and APX activities must not only be sufficiently high, but also balanced, so that H_2O_2 generated by the action of SOD upon superoxide radicals is eliminated by APX [7]. Some experimental evidence has suggested that APX is less stable than SOD under oxidative stress conditions generated by methyl viologen in some crops [13,20]. APX is present predominantly in the cytosol and organelles and plays a major role in H_2O_2 elimination through the Halliwell-Asada pathway. In the presence of paraquat, illuminated

chloroplasts start to accumulate H_2O_2 within minutes [28] and APX is the first enzyme to become inactivated after short light exposure [13,14]. Even in the absence of MV, the addition of H_2O_2 in the dark inactivates APX [33]. On the other hand, enzymes like SOD, DHAR and GR lose their activities only after a long period of light exposure in the presence of MV [34]. These data show the higher susceptibility of APX compared to other antioxidative enzymes. Our results also substantiate the higher susceptibility of APX, compared to another antioxidative enzyme, SOD, to MV-induced oxidative stress in sugarcane.

Our results did not show evident induction of APX activity in MV-stressed leaves except at 2 mM MV concentration. This fact could have been due to the high level of MV toxicity in these sugarcane leaves, generating oxidative deterioration of biological macromolecules, including proteins and nucleic acids. Donahue et al. [35] observed that an increase in mRNA levels of APX induced by paraquat did not reflect a correspondent increase in APX activity. Similar results were found in isolated tobacco chloroplasts, where APX activity did not show any significant increase by induced oxidative stress [14].

Transformed plants containing single transgenes to express antioxidant enzymes (e.g., SOD, APX) have been developed and studied. However, manipulation of a single antioxidant enzyme has provided only little improvement to stress tolerance. The improvement in oxidative stress tolerance due to high expression of SOD has not been similar in transgenic plants [36]. As an example, McKersie et al. [37,38] found improvements in oxidative stress tolerance, whereas Payton et al. [39] found no improvements. Kwon et al. [40] reported an enhanced tolerance of transgenic tobacco plants that expressed both SOD and APX in chloroplasts against oxidative stress induced by paraquat. The reason for these differences is due to the complexity of the ROS detoxification system, since changing a single antioxidant enzyme may not change the overall capacity of the antioxidant pathway.

At a non-toxic level of MV (2 mM) the activities of SOD and APX displayed contrasting responses when compared to toxic levels ($\geqslant$4 mM) in sugarcane leaves. Indeed, after 24 h, the activity of SOD remained unchanged, whereas the activity of APX increased significantly. In contrast, at toxic levels of MV, the activity of APX decreased abruptly, along with soluble protein content, whereas SOD activity was strongly resistant to oxidative damage. Furthermore, oxidative damage, based on photochemical damage, lipid peroxidation and chlorophyll and soluble protein content were inversely related to APX activity. These results strongly suggest that under mild oxidative stress due to excess superoxide produced by MV, the SOD-APX defense system was efficient whereas under acute stress, the labile nature of APX probably contributed to oxidative damage, which was possibly caused by excess H_2O_2.

In conclusion, the present data strongly suggest that the major MV-induced oxidative damages in sugarcane leaves might be related to excess H_2O_2 caused by an imbalance between SOD and APX activities, where APX was limiting due to decreased activity. An important early indication of this oxidative stress was the observed reduction in photochemical activity.

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