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Sunflower cotyledons cope with copper stress by inducing catalase subunits less sensitive to oxidation

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ABSTRACT

Copper is an essential trace element for living organisms, in excess, can be toxic to the cell because of its capacity to generate reactive oxygen species (ROS). Catalase (CAT) catalyzes the dismutation of hydrogen peroxide into water and dioxygen and in plants it is located in peroxisomes and glyoxysomes. Different metals can induce changes in CAT activity, but the mechanism underlying its changes is unclear. After 4 h of treatment with 5 and 10 μM $CuCl_2$ a decrease in the specific CAT activity was detected in sunflower cotyledons of post-germinative heterotrophic seedlings. At 8 h of treatment, 5 μM Cu^{2+} produced an induction of CAT activity while only a complete recovery to control values was observed for 10 μM Cu^{2+} treated seedlings. These activity variations were not related to the level of CAT protein expression, but they did correlate with the oxidative state of the CAT protein. This indicates that the mechanism of CAT inactivation by Cu^{2+} involves oxidation of the protein structure. The level of the mRNA of *CATA3* and *CATA4* increased with the presence of the metal after 4 h of exposure. These CAT genes code for the synthesis of CAT subunits less sensitive to oxidation, which would prevent the copper-induced oxidative inactivation of CAT.

Introduction

Copper is considered an essential trace element for the living organisms. As part of the plant cell components it plays a vital role as a prosthetic group of many proteins involved in redox reactions, like plastocyanin, cytochrome *c* oxidase or superoxide dismutase, and it is also required by the ethylene receptor for proper signaling [1,2].

At the same time that the amount of copper available to the organism is critical for its life, an excess of this metal can be toxic for the cell. Copper is a redox active metal that has the ability to catalyze, via the so-called Fenton reaction, the generation of reactive oxygen species (ROS) [3]. ROS are products obtained from the partial reduction of oxygen molecule that include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) or hydroxyl radical (HO•) [4]. To cope with ROS, organisms have developed a protective system that includes enzymatic (superoxide dismutase, catalase, glutathione reductase, ascorbate peroxidase) and non-enzymatic antioxidant defenses (glutathione, ascorbic acid) [4]. An increased concentration of oxidant species and/or a decrease in the antioxidant level could lead to the generation of oxidative stress.

ROS can cause oxidative modification of cellular components in plants. This type of damage will lead to an altered structure and loss of functionality of macromolecules [5]. Proteins, as well as other molecules such as lipids, are the most common cellular targets of the oxidative species; protein oxidation may result in modification of their enzymatic and binding properties and lead to diverse functional changes. Carbonylation of specific amino acid residues is the most common oxidative protein modification, and it is characterized as an irreversible and unrepairable process [6].

Catalase (H_2O_2:H_2O_2 oxidoreductase; EC 1.11.1.6) is one of the most important antioxidant enzymes. It is an iron porphyrin tetrameric protein found in all aerobic organisms that catalyzes the dismutation of H_2O_2 to O_2 and H_2O. In plant cells catalase decomposes H_2O_2 derived from β-oxidation of fatty acids during the heterotrophic, post-germinative growth phase of oil-rich seedlings (such as sunflower), within specialized peroxisomes called glyoxysomes. In photoautotrophic cells, catalase degrades H_2O_2 produced during the photorespiration within leaf-type peroxisomes, which are derived from glyoxysomes [7]. Moreover, catalase confers resistance to oxidative stress by detoxification of ROS that could be produced within the cell by adverse environmental conditions, such as drought [8,9], temperature [10], or UV-B radiation [11]. On the other hand, due to the fact that H_2O_2 is recognized as a second messenger and catalase acts as a key regulator of this species, this enzyme could act as mediator in the signal transduction pathway [12].

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Multiple forms of the CAT enzyme encoded by a small unlinked nuclear gene family have been reported for many higher plants. When CAT subunit polypeptides encoded by distinct genes are simultaneously expressed in the same cell, heterotetramers of CAT may be formed. In sunflower (*Helianthus annuus* L.) at least eight isoforms have been identified: CAT1–CAT8. According to studies of the composition of the protein, these eight isoforms can be divided into two groups. The first group (CAT1 through CAT5) arises as the consequence of interactions between four 55 and 59 kDa subunits in various proportions, whereas the second group (CAT6 through CAT8) contains exclusively 55 kDa subunits [13]. The biogenesis of the subunits is controlled by four different genes (*CATA1–CATA4*). *CATA1* and *CATA2* are assumed to code for subunits of 55 kDa, whereas *CATA3* and *CATA4* code for subunits of 59 kDa [8]. The presence of various isoforms may reflect the multiple functions of this enzyme, but the physiological roles of some isoforms remain yet uncertain.

Although numerous reports describe the impact of the excess of metals on plant catalase activity as part of the antioxidant response, the mechanism underlying its activity changes is unclear. Due to excess of copper is associated, at least in part, to the generation of oxidative stress, we performed this study in terms of activity, amount and oxidation of CAT protein and CAT transcript levels during the heterotrophic post-germinative stage of sunflower seeds.

Materials and methods

Plant material and growing conditions

Sunflower seeds (*H. annuus* L., cv DK3820, supplied by Dekalb, Buenos Aires, Argentina) were surface sterilized with 20% (v/v) sodium hypochlorite solution (55 g L^{-1} available chlorine) for 10 min and washed several times with distilled water. After that, seeds were soaked in demineralised water in a rotary shaker in dark conditions, at 100 rpm and 24 ± 2 °C. After 48 h of growing, seed pericarps were removed and naked seedlings with 1 cm of root length were selected and used for the assay. Eight seedlings were transferred to flasks with 30 mL of demineralised water (control) or with solutions containing 5 and 10 μM $CuCl_2$. The flasks were incubated at 24 ± 2 °C in darkness. After 4 and 8 h of incubation, germination seedlings were gently washed with distilled water; cotyledons were removed and used for the determinations. Experiments were repeated three times with five replicates per treatment.

Carbonyl group content

Protein oxidation was measured as the total carbonyl group content by reaction with 2,4-dinitrophenylhydrazine (DNPH) [14]. Extracts were prepared from cotyledon tissue (1 g), homogenized in extraction buffer (10 mL) consisting of 50 mM phosphate buffer (pH 7.4), 120 mM KCl, 1 mM EDTA and 0.1 g polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 10,000 × *g* for 20 min. Aliquots of the supernatant containing at least 0.5 mg protein were incubated with 0.03% Triton X-100 and 1% streptomycin sulfate for 15 min to remove the nucleic acids. After centrifugation at 8500 × *g* for 10 min, 500 μL of the supernatant were mixed with 500 μL of 10 mM DNPH in 2 M HCl for 1 h at room temperature. Blank samples were incubated in 2 M HCl. Proteins were precipitated with 20% (w/v) TCA, the pellets were washed three times with ethanol:ethylacetate (1:1) and finally dissolved in 6 M guanidine hydrochloride in 20 mM potassium phosphate buffer (pH 2.3), adjusted with trifluoroacetic acid. Absorption at 380 nm was measured and carbonyl group content was calculated using a molar absorption coefficient of 22,000 M^{-1} cm^{-1}.

Determination of CAT activity

Extracts for determination of CAT activity were prepared from 0.5 g of cotyledon tissue and homogenized under ice-cold conditions in 5 mL of extraction buffer containing 50 mM K-phosphate buffer (pH 7.4), 1 mM EDTA, 1 g PVP, and 0.5% (v/v) Triton X-100 at 4 °C. Homogenates were centrifuged at 10,000 × *g* for 30 min and the supernatant fraction was used for the assay. CAT activity was determined in the homogenates by measuring the decrease in absorbance at 240 nm in a reaction medium containing 50 mM potassium phosphate buffer (pH 7.2) and 2 mM H_2O_2. The pseudo-first order reaction constant ($k' = k \times [CAT]$) of the decrease in H_2O_2 absorption was determined and the CAT content was calculated using $k = 4.7 \times 10^7$ M^{-1} s^{-1} [15].

Western blot of catalase, immunoprecipitation and immunochemical detection of catalase carbonyl groups

Extracts obtained in the same extraction buffer as described above supplemented with 2 mM cysteine, 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.04% 2-mercaptoethanol (50 μg of protein) were subjected to electrophoretic analysis using 12% (w/v) SDS-polyacrylamide gel (PAGE) [16]. This gel was electrotransferred onto PVDF membranes and polyclonal antibodies raised against cotton seed catalase (generously provided by Dr Trelease) were employed to detect catalase protein. Bands were subsequently visualized using a secondary goat antibody conjugated with horseradish peroxidase and stained using 3,3-diaminobenzidine (DAB) as substrate. Membranes were photographed with Fotodyn equipment, and analyzed with GelPro software.

For the immunoprecipitation and immunochemical detection of catalase carbonyl groups, proteins (100 μg) were derivatized with DNPH as described above, but in this case proteins were dissolved in 100 mM buffer sodium phosphate (pH 7.5) and then were separated by affinity chromatography. Antibodies anti-DNP (50 μL) were linked to cyanogen bromide activated Sepharose 4% agarose matrix (100 mg) from Sigma–Aldrich (St Luis, USA). Samples were incubated overnight at 4 °C with an excess of anti-DNP-agarose resin and then centrifuged for 5 min at 10,000 × *g*. Resin beads were washed 3 times with Tris-buffered saline (TBS), with pellets resuspended in 100 mM glycine–HCl (50 μL, pH 2.5). After centrifugation, the pellets were discarded, the pH of the supernatants adjusted to 6.8 with 0.5 M Tris–HCl buffer (5 μL, pH 8.8) and used for immunodetection of catalase. DNPH derivatized proteins were separated by 12% (w/v) SDS-PAGE. After electrotransfer of the proteins to nitrocellulose membranes, the catalase was detected, photographed and analyzed as previously described.

Semiquantitative RT-PCR

Total RNA was extracted from cotyledon tissue using a modified TRIzol (Invitrogen, Carlsbad, CA) procedure. The RNAs were then treated with DNase I (Promega). They were then converted to cDNAs with random primers using the RevertAid™ M-MuLV Reverse Transcriptase (Fermentas). Primers for PCR amplifications are described in Table 1. PCRs were performed using a programmable Termocycler T 18 (Ivema) at annealing temperatures of 54 °C for 18S and 50 °C for *CATA*s. For an accurate comparison and quantification of the transcript levels, the exponential phase of PCR amplifications was determined by establishing the number of PCR cycles where the products exhibit an exponential phase: 19 cycles for 18S PCR products and 31 cycles for *CATA*s PCR products. The PCR products were electrophoresed through 1.2% agarose and visualized with ethidium bromide. Gel was photographed with Fotodyn, analyzed with GelPro software. The band intensity was

Table 1
Primers used for amplification of *CATA*s and GenBank accession number.

Gene	Primer sequences	Accession number
CATA1	Forward 5′-CTTCCCGCTTGAATGTGAAG-3′ Reverse 5′-CCGATTACATAAACCCATCATC-3′	L28740
CATA2	Forward 5′-CTTCCCGCTTGAATGTGAAG-3′ Reverse 5′-AGCATGTCAAGAACATTATCAC-3′	AF243517
CATA3	Forward 5′-GGGCAGAAGATAGCATCACG-3′ Reverse 5′-AATACATGAACGGAAGCATCA-3′	AF243518
CATA4	Forward 5′-GGGCAGAAGATAGCATCACG-3′ Reverse 5′-GTAACTCACTCCACATATCGAC-3′	AF243519

expressed as arbitrary units (assuming control value equal to 1), based on absolute integrated optical density of each *CATA* band. 18S serves as an internal standard.

Determination of protein

Protein concentration was determined according to Bradford [17] using bovine serum albumin as standard.

Statistics

Values are expressed as mean ± S.E.M. Differences among treatments were analyzed by one-way ANOVA, taking $P < 0.05$ as significant according to Tukey's multiple range test.

Results

Effect of copper on cotyledons protein oxidation

Carbonyl group content is widespread used as a parameter of protein oxidation. Carbonyl group content remained unchanged among cotyledons of control and Cu-treated plants after 4 h of treatment. However, this parameter showed a significant dose-dependent increase at 8 h of treatment. By this time, carbonyl group content increased 39 and 77% for 5 and 10 μM $CuCl_2$, respectively, with respect to control values (Fig. 1).

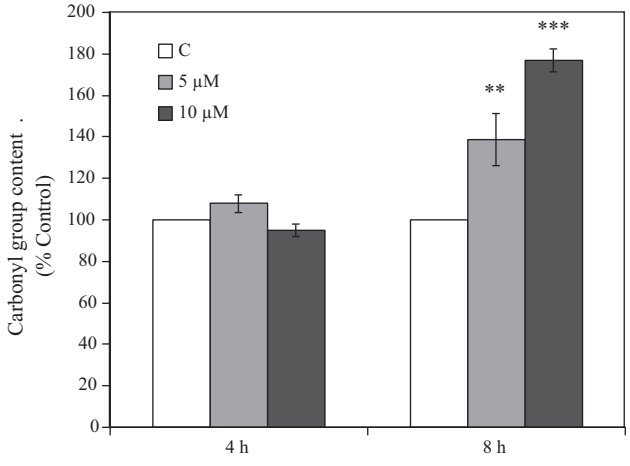

Fig. 1. Effect of copper on sunflower cotyledons protein oxidation. Two-day sunflower naked seedlings were incubated in demineralised water (control) or in solutions containing 5 and 10 μM $CuCl_2$. After 4 and 8 h of treatment cotyledons were removed and used for the determination of carbonyl group content. Data are expressed as percent of control values (means ± S.E.M.). Asterisks indicate significant differences (**$P < 0.01$, ***$P < 0.001$) according Tukey's multiple range test.

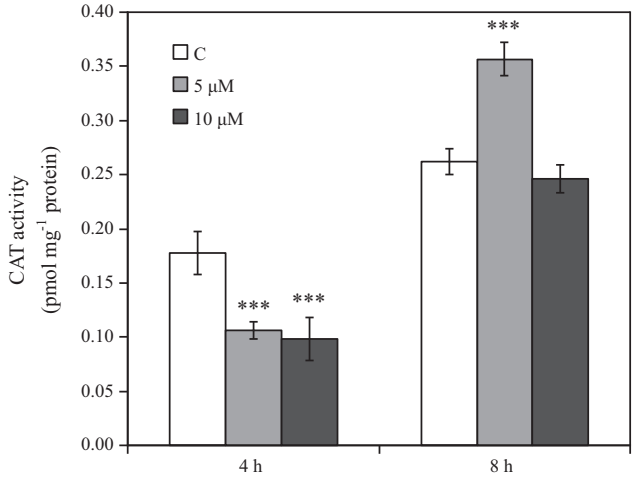

Fig. 2. Effect of copper on sunflower cotyledons CAT activity. Two-day sunflower naked seedlings were incubated in demineralised water (control) or in solutions containing 5 and 10 μM $CuCl_2$. After 4 and 8 h of treatment cotyledons were removed and used for CAT activity determination. Values are means ± S.E.M. Asterisks indicate significant differences (***$P < 0.001$) according Tukey's multiple range test.

Effect of copper on specific catalase activity

Sunflower cotyledons of Cu-treated seedlings showed a decrease in the specific CAT activity (40 and 45% of decline for 5 and 10 μM Cu^{2+}, respectively, related to control value) at 4 h of treatment. After that, the lowest Cu^{2+} concentration used increased specific CAT activity 40% over the control seedlings, but only a recovery to control values was observed for 10 μM Cu^{2+} treated plants (Fig. 2A).

Catalase protein abundance and oxidation

Western blot analysis revealed that under both copper concentrations used, total CAT protein abundance remained similar to controls after 4 or 8 h of treatment (Fig. 3A). Fig. 3B shows the state of oxidation of CAT protein. Interestingly, even though there was not an increase of total carbonyl group content after 4 h of treatment (Fig. 1), at this time copper produced CAT protein oxidation. The increment of CAT protein oxidation was different on the metal concentration assayed. After 8 h of 5 μM Cu^{2+} treatment, the level of CAT protein oxidation returned to the value observed in control cotyledons while in 10 μM Cu^{2+} treatment a significant decrease was detected (Fig. 3B).

Effect of copper on CATAs transcripts

All the CAT transcripts (*CATA1*, *CATA2*, *CATA3* and *CATA4*) described for sunflower were analyzed and detected in cotyledons of control plants. Under copper treatment, *CATA1* and *CATA2* levels remained unaffected in cotyledons of treated plants after 4 h of metal exposure. On the other hand, mRNA levels of *CATA3* and *CATA4* were enhanced in response to the presence of the metal; the increment detected was close to 2.5 fold over the control (Fig. 4).

Discussion

Catalase is a very important enzyme in all oil seeds and it has been proved that its activity is closely related to the germination rate in sunflower [8]. During the heterotrophic sunflower seedling growth, copper treatment rapidly declined CAT activity in cotyledons, although there were not visible symptoms of metal toxicity, such as growth reduction or root apices browning. Afterwards, a

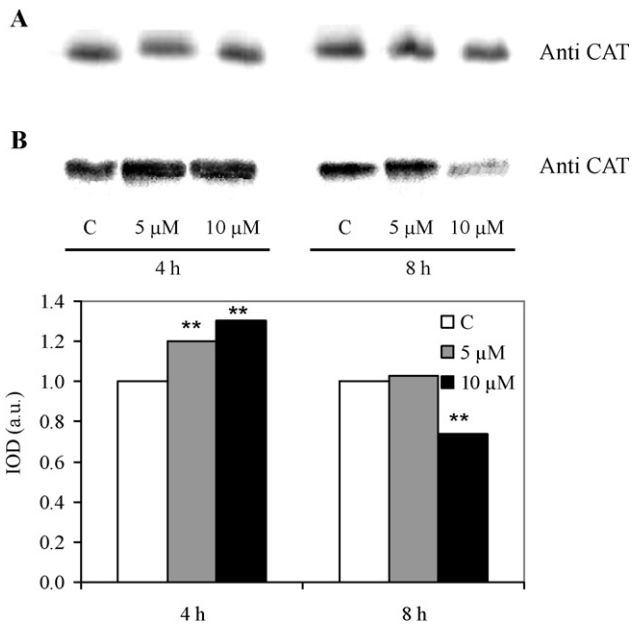

Fig. 3. Effect of copper on catalase protein and identification of catalase oxidized protein. (A) Western blotting of 12% (w/v) SDS-PAGE. (B) Western blotting of SDS-PAGE (12% w/v) after derivatization of proteins with 2,4 DNPH, followed by immunoseparation with anti-DNP. Western blotting was performed using an anti-cotton seed catalase antibody and bands were visualized as described in "Materials and methods" section and expressed in arbitrary units (a.u.) assuming control value equal to 1, based on absolute integrated optical density (IOD) of each band. Asterisks indicate significant differences with respect to control at $^{**}P < 0.01$, according to Tukey's multiple range test. The data shown are representative of three experiments with a total of four to five samples/group.

recovery of the enzyme activity was observed. These activity variations were not related with the level of the CAT protein expression, but they correlated with the oxidative state of the CAT protein. Protein oxidation is widespread and often used as a diagnostic marker for oxidative stress. Protein can be the target of ROS which can produce carbonylation of their amino acids. As a consequence, the protein may lose their function [18]. Thus, the decrease observed

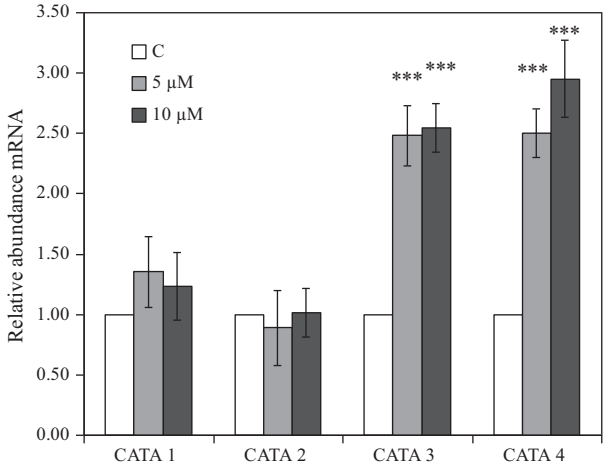

Fig. 4. Effect of copper on CAT transcripts accumulation in sunflower cotyledons. Two-day sunflower naked seedlings were incubated in demineralised water (control) or in solutions containing 5 and 10 μM $CuCl_2$. After 4 h of treatment cotyledons were removed, and RT-PCR for *CATA*s was performed as described in "Materials and methods" section. Relative mRNA values were calculated as the ratio *CATA*/*18S*. Data are expressed as arbitrary units (assuming control value equal to 1), based on absolute integrated optical density of each band. Asterisks indicate significant differences with respect to control at $^{***}P < 0.001$, according to Tukey's multiple range test. The experiment was repeated three times and a representative image is presented.

in CAT activity under copper treatment could be partly due to an oxidative inactivation of the enzyme. A similar result was observed with cadmium treatment, a metal also associated with the production of oxidative stress [19]. In our experimental work we could observed that CAT protein oxidation precedes the bulk of protein oxidation generated by copper because at 4 h not did shown total oxidized protein however CAT protein oxidation was present. The fact that total protein carbonylation may not reflect the oxidation of catalases suggests a particular sensitivity of this protein to the oxidizing conditions.

The effect of an excess of copper on catalase gene expression was studied at the level of steady-state mRNA abundance as estimation of antioxidant gene activation. The four CAT transcripts were detected in the cotyledon tissue in the post-germinative heterotrophic seedling growth, but only overexpression of *CATA3* and *CATA4* was detected under copper treatment. Up-regulation of these genes in sunflower cotyledons seems to be a direct response to the increase in the oxidative stress conditions. This observation is in accordance with other reports where oxidative stress is related to an increase in CAT transcripts. In the development and germination of scutella of maize, *CAT1* expression was highly induced by ROS [20]. Recently, it was demonstrated an induction of *CAT3* in Arabidopsis in response to alternative catabolic substrates utilization during sucrose starvation-induced oxidative stress [21].

The increase in CAT activity observed at 8 h in cotyledons of copper treated plants probably derived from the differential transcription of all CAT genes. As it was observed by Luna et al. [9] the fact that increased accumulation of mRNAs do not result in more CAT protein, shows the complex regulation of CAT mRNA, particularly at the level of translation. In wheat leaves experiencing water deficits the increase in CAT activity observed could not be explained by enhanced transcription [9]. Mylona et al. [20] indicated that the differential expression of CAT genes involves both transcriptional and post-transcriptional regulation, which is superimposed on the responses of these genes to elevated levels of ROS. According to the results presented in this work, it is not possible to draw conclusions about the relative contribution of *CATA3* and *CATA4* genes on the increase of CAT activity observed under copper treatment.

The *CATA*s which were accumulated during the copper treatment codes for subunits of 59 kDa [8]. Grotjohann et al. [22] demonstrated that sunflower catalase isoforms differ in their sensitivity and the residual activity to *in vitro* conditions of oxidative photoinactivation, indicating a different resistance to oxidative damage of the subunits of 55-kDa and 59-kDa. In photo-oxidative conditions, the group of isoforms containing both subunits was less photosensitive than the isoforms composed only by 55-kDa subunit. In Cu-treated seedlings, the reduction in the state of CAT protein oxidation observed at 8 h respect to those detected at 4 h of metal treatments shows that isoforms less sensitive to oxidation are being synthesized. The isoform changeover in sunflower could be physiologically related to the acclimatization to the oxidative condition exerted by copper. Engel et al. [23] confirmed that *HNNCATA3* coded for a light-insensitive catalase and only those few unique amino acid substitutions that occur in strictly conserved positions of *CATA3* of *H. annuus* can be expected to contribute to the extraordinary resistance to photoinactivation of catalase. Induction of transcription of *CATA3* and *CATA4* gene during copper treatment derived in CAT isoforms enriched in the 59-kDa subunit and therefore less sensitive to the oxidative damage. It should be noted that *CATA3* and *CATA4* codes for subunits characteristics of the CAT present in the *core*, whereas *CATA1* and *CATA2* codes for subunits localized in the matrix of the peroxisomes.

In conclusion, copper, a redox active metal, decreases CAT activity in cotyledon tissue in the post-germinative heterotrophic seedling and the mechanism of this inactivation involves the oxidation of CAT protein. The conditions exerted by the presence of

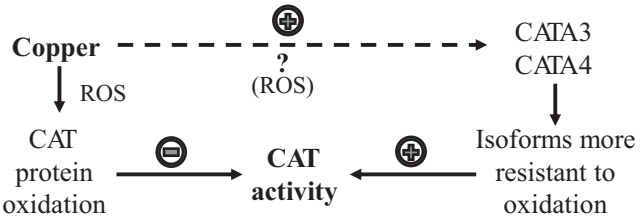

Fig. 5. Proposed model illustrating the effect of Cu on sunflower catalase. Copper increases reactive oxygen species (ROS) that oxidize CAT protein. These oxidative modifications alter its function decreasing its activity. As consequence, CAT genes that code for subunits less sensible to oxidation are induced, and thus prevent the decrease in the enzyme activity.

the metal induce the expression of CAT transcripts that lead to the synthesis of CAT isoforms less sensitive to oxidation, which prevent the enzyme inactivation (Fig. 5). The present study contributes to a major comprehension of the effect of copper on the plant antioxidant system.

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