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ACTIVITIES OF ANTIOXIDATIVE ENZYMES AND SENESCENCE IN DETACHED *CUCURBITA PEPO* UNDER CU- AND OXIDATIVE STRESS BY H_2O_2

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The activities of antioxidative enzymes were determined in detached leaves of *Cucurbita pepo* exposed to either Cu stress or oxidative stress by hydrogen peroxide. Cu reduced the activities of glutathione reductase (GR), catalase (CAT), and ascorbate peroxidase (APOD), dehydroascorbate reductase (DHAR) but increased the activity of superoxide dismutase (SOD). On the other hand, hydrogen peroxide decreased the activities of GR, APOD, SOD, and DHAR but increased the activity of catalase. The effects of Cu or hydrogen peroxide stress on the different tested enzymes were minimized by pretreatment with ascorbic acid (ASA), reduced glutathione (GSH) or both. Cu stress increased the levels of endogenous hydrogen peroxide and malondialdehyde (MDA) but decreased the levels of protein, chlorophyll a and chlorophyll b. Cu-promoted senescence and the increase of MDA level were inhibited by ASA, GSH, benzoic acid (BA) or thiourea (TU). Hydrogen peroxide stress increased MDA content but decreased the levels of endogenous hydrogen peroxide, protein, chlorophyll a and chlorophyll b. Hydrogen peroxide-induced senescence and the increase in MDA content were inhibited by the various tested free radical scavengers.

Soils occasionally contain phytotoxic amounts of metals, but more frequently, they accumulate them as a consequence of industrial and agricultural activity [1].

Cu is important in various biochemical processes, but at toxic concentrations it interferes with numerous physiological processes [2, 3]. It has been investigated that Cu mediated free radicals' formation in detached leaves [4] and isolated chloroplasts [5, 6].

Hydrogen peroxide (H_2O_2) is a constituent of oxidative plant metabolism. It is a product of peroxisomal and chloroplastic oxidative reactions [7]. H_2O_2 is an active oxygen species that can react with superoxide radicals to form more reactive hydroxyl radicals in the presence of trace amounts of Fe or Cu [8].

Hydrogen peroxide, hydroxyl radical and superoxide, are reactive oxygen species [9]. Accumulation of these active oxygen species may cause peroxidation of membrane lipids and inactivation of enzymes [10].

The protective mechanisms adapted by plants to scavenge free radicals and peroxides include several antioxidative enzymes such as SOD, APOD, GR, CAT and DHAR. These enzymes are important components in preventing the oxidative stress in plants as is based on the fact that activity of one or more of these enzymes is generally increased in plants when exposed to stressful condition [11].

SOD catalyzes the dismutation of superoxide radical to molecular oxygen and H_2O_2. The produced hydrogen peroxide is then detoxified by CAT or APOD [12]. APOD reduces H_2O_2 to water, with ascorbate as an electron donor [13]. GR plays a part in the control of endogenous H_2O_2 through an oxido-reduction cycle involving glutathione and ascorbate [14].

The aim of the present investigation was to investigate the changes in the activities of antioxidative enzymes SOD, APOD, GR, CAT and DHAR; lipid peroxidation; endogenous H_2O_2 level and accelerated senescence in detached leaves of *Cucurbita pepo* under excess Cu- and oxidative-stress by H_2O_2.

MATERIALS AND METHODS

Seeds of *Cucurbita pepo* were surface sterilized in 10 % (v/v) sodium hypochlorite for 10 min, soaked in running tap water for 24 h, and then germinated between paper towels, moistened with distilled water in sterilized plastic trays. The trays were covered and incubated for 24 h in the dark at 25°C. Seeds with well-grown roots were then supported on plastic grids through which the roots projected. The grids were floated in small plastic bowls containing half-strength Johnson's modified nutrient solution (pH 4.2). The nutrient solution was replaced every three days. The apical 3-cm segments excised from the leaves of 10-day old seedlings were used. The segments were floated in a Petri dish containing 20 ml of test solutions. Incubations were carried out at 25°C (50 μmol $m^{-2}s^{-1}$) in light.

Crude preparation was obtained from leaves of 10-old seedlings according to the method of Chen et al. [15]. Leaves were homogenized with 0.1 M sodium phosphate buffer (pH 6.8) in a chilled pestle and mortar. The homogenate was centrifuged at 12,000 g for 20 min and the resulting supernatant was used for determination of the enzyme activity. The whole extraction procedure was carried out at 4°C. The results are the means of three determinations ± s.e.

SOD and DHAR were measured by the method of Wang *et al*. [16]. APOD was measured by the method of

Converso and Fernandes [17]. GR was assayed according to Schaedle and Bassham [18]. CAT was assayed according to Sadasivam and Manickam [19].

Protein content was determined according to Lowry et al. [20] using bovine serum albumin as standard. Chlorophyll a and b were determined by the method of Oliveira et al. [21]. Lipid peroxidation was measured as Malondialdehyde (MDA) by the thiobarbituric acid (TBA) reaction as described by Ranieri et al. [22]. H_2O_2 was measured colorimetrically according to Jana and Choudhuri [23].

RESULTS AND DISCUSSION

In the present study $CuSO_4$ increased the activity of SOD in detached *Cucurbita* leaves compared to the control (Fig. 1). The increase of SOD activity can be considered as an indirect evidence for enhanced production of free radicals under Cu stress. In support, it has been reported that excess Cu increased the activity of SOD in plant tissues [24, 25, 15]. However, Chen and Kao [26] showed that SOD activity did not seem to be affected by excess Cu^{2+} ions. $CuSO_4$ decreased the activities of APOD, GR, CAT and DHAR compared to the control (Fig. 1). These results are consistent with those of Chen and Kao [26]. Mazhoudi et al. [25] reported that Cu did not affect CAT and APOD activities. Such a variation in response of these enzymes to Cu stress could be due to the variability of plant species in producing free radicals [25].

GR catalyzes the reduction of oxidized glutathionine (GSSG) in an NADPH-dependent reaction. GR, therefore, plays an essential role in the protection of chloroplasts against oxidative damage by maintaining a high GSH/GSSG ratio. In the present work, GR activity is decreased in detached *Cucurbita* leaves exposed to excess $CuSO_4$ (Fig. 1). These results are in harmony with those of Shainber et al. [27] and suggest a decrease in GSH/GSSG ratio. This would explain why $CuSO_4$-treatment resulted in oxidative damage in detached *Cucurbita* leaves. Treatment of detached *Cucurbita* leaves with H_2O_2 decreased SOD, APOD, GR and DHAR activities but increased the activity of CAT (Fig. 1). These results are in agreement with those of Lin and Kao [28].

When detached *Cucurbita* leaves were pretreated with the free radical scavengers ascorbic acid (ASA, destruction of active oxygen species, particularly H_2O_2) and reduced glutathione (GSH, scavenger of free radicals) then treated with 10 mM of $CuSO_4$ (Table 1) or H_2O_2 (Table 2) for 12 h they were able to prevent the decrease in the enzyme activities. GSH was better scavenger than ASA and a mixture of the two compounds showed the best prevention. Lin and Kao [28] reported similar results. GSH and ASA are the main antioxidants and are present in plant leaves [29]. GSH can react with singlet oxygen and hydroxyl radicals and protects the thiol groups of enzymes [30].

Senescence of detached leaves is characterized by a decrease in protein and chlorophyll levels [31]. Treatment of detached *Cucurbita* leaves with 10 mM of either $CuSO_4$ or H_2O_2 resulted in decreasing the protein levels (Fig. 2).

Excess Cu may replace other metals in metalloprotein or may interact directly with SH-groups of proteins [32]. Cu-induced free-radical formation may also cause protein damage [2, 33].

The chlorophyll a and b contents were decreased in detached *Cucurbita* leaves treated with either $CuSO_4$ or H_2O_2 (Fig. 3). Lin and Kao [28] and Chen and Kao [26] also reported the reduction in chlorophyll levels under Cu

Table 1

Effect of ascorbic acid (ASA) and glutathione (GSH) at 10 mM on the activities of antioxidative enzymes in detached *Cucurbita* leaves treated with 10 mM $CuSO_4$. The enzyme activities were measured after 48 h of treatment. Values are means ± S.E.

Treatment	Enzymeactivity (Ug^{-1} FW)				
	SOD	APOD	GR	CAT	DHAR
Control	61.4±0.7	47.5±0.4	33.4±0.5	13.2±0.1	15.3±0.2
$CuSO_4$	112.2±0.5	17.3±0.9	19.7±0.7	4.4±0.2	6.6±0.1
ASA+$CuSO_4$	119.3±0.4	25.5±0.6	23.6±0.4	6.5±0.3	8.4±0.2
GSH+$CuSO_4$	124.4±0.7	30.3±0.8	27.5±0.8	10.8±0.2	10.8±0.1
ASA+GSH+$CuSO_4$	128.2±0.3	43.2±0.5	31.3±0.6	12.2±0.1	14.5±0.2

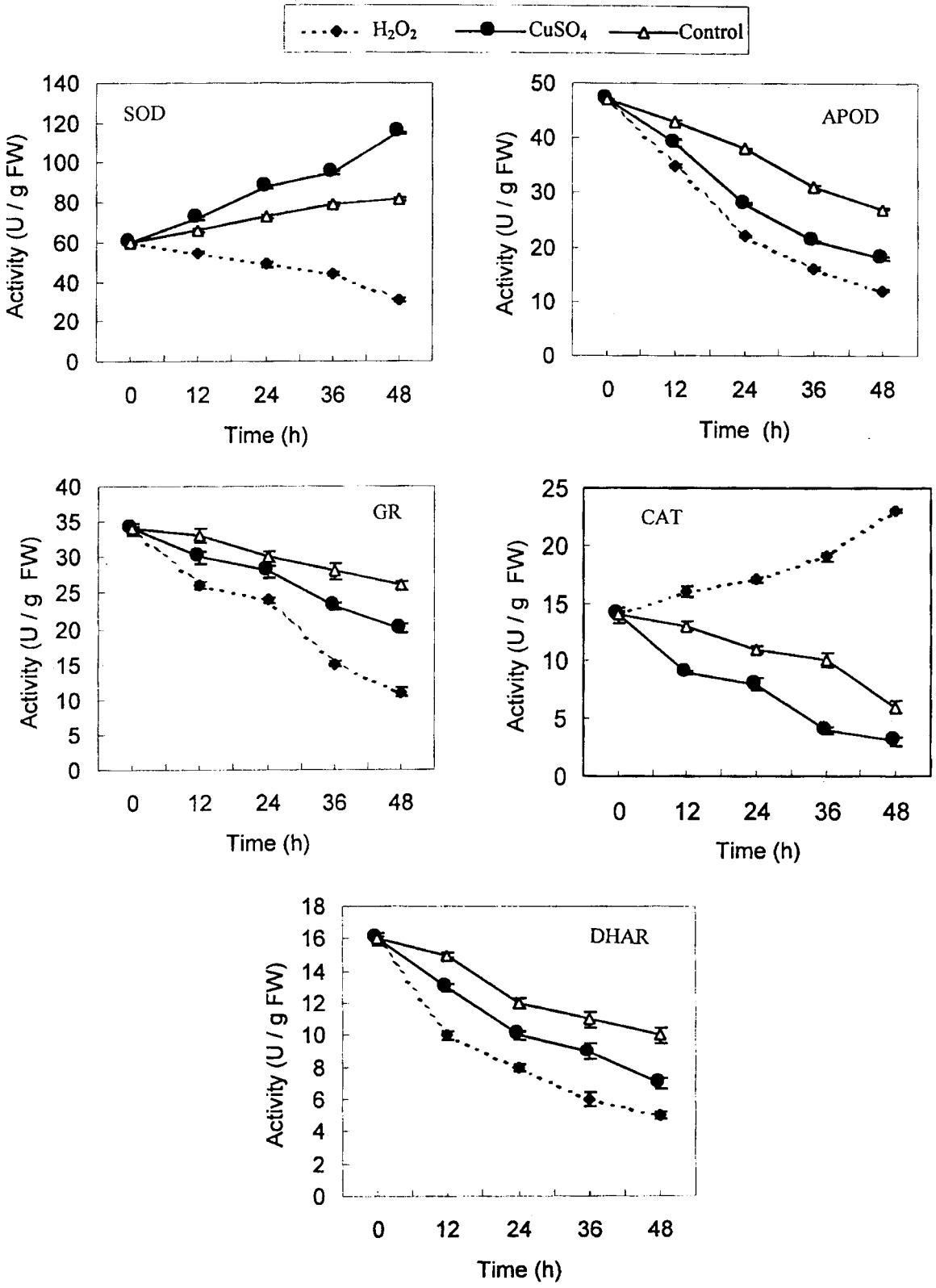

Fig. 1. Effect of $CuSO_4$ and H_2O_2 at 10 mM on the activities of SOD, APOD, GR, CAT and DHAR in *Cucurbita* leaves

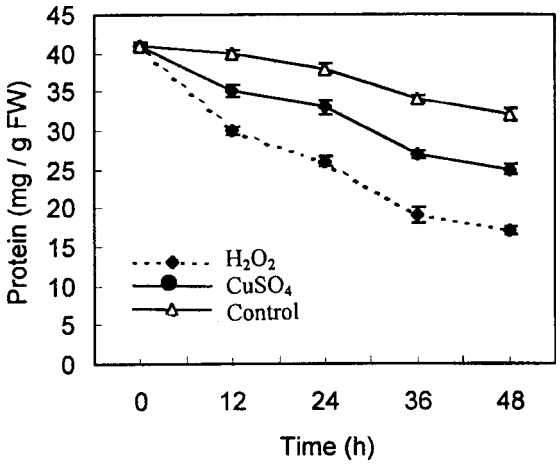

Fig. 2. Effect of $CuSO_4$ and H_2O_2 at 10 mM on protein levels in detached *Cucurbita* leaves

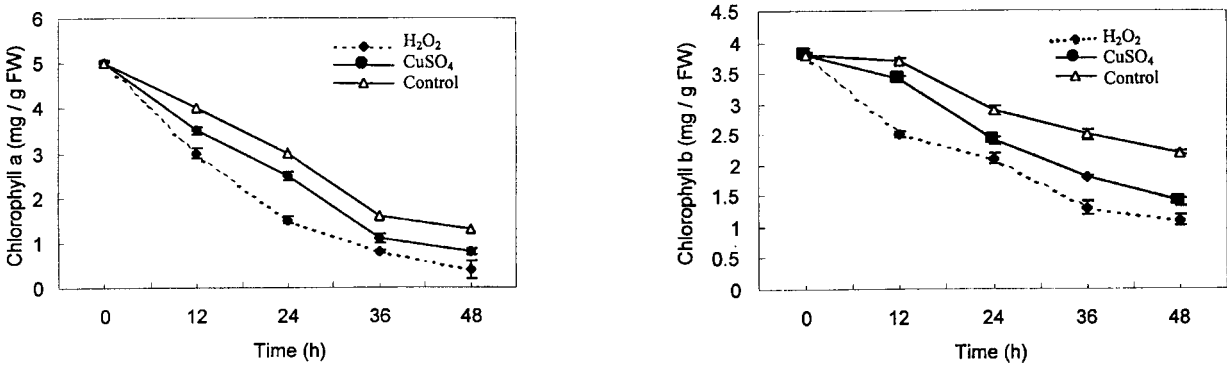

Fig. 3. Effect of $CuSO_4$ and H_2O_2 at 10 mM on the levels of chlorophyll (*a*) and chlorophyll (*b*) in detached *Cucurbita* leaves

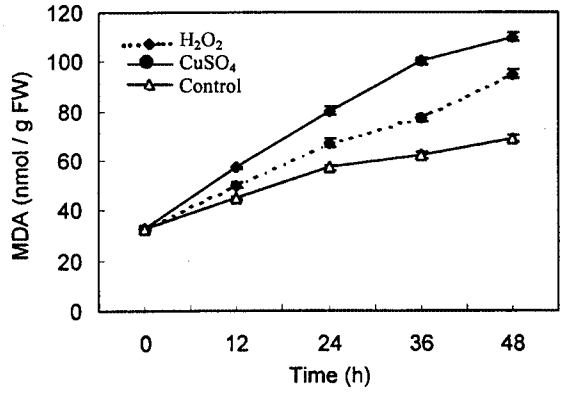

Fig. 4. Effect of $CuSO_4$ and H_2O_2 at 10 mM on MDA in detached *Cucurbita* leaves

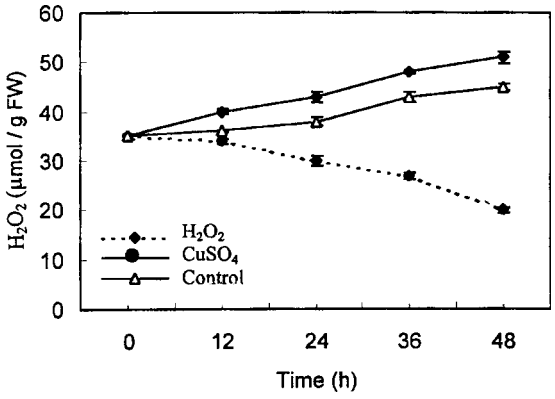

Fig. 5. Effect of $CuSO_4$ and H_2O_2 on the endogenous H_2O_2 in detached *Cucurbita* leaves

Table 2

Effect of ascorbic acid (ASA) and reduced glutathione (GSH) at 10 mM on the activities of antioxidative enzymes in detached *Cucurbita* leaves treated with 10 mM H_2O_2. The enzyme activities were measured after 48 h of treatment. Values are means ± S.E.

Treatment	Enzymeactivity (Ug^{-1} FW)				
	SOD	APOD	GR	CAT	DHAR
Control	59.4±0.5	45.6±0.6	32.2±0.6	14.6±0.1	16.3±0.3
H_2O_2	30.3±0.4	12.3±0.8	11.5±0.7	23.5±0.3	5.6±0.2
ASA+H_2O_2	35.8±0.8	16.7±0.4	14.7±0.4	27.6±0.2	8.4±0.1
GSH+H_2O_2	49.4±0.6	22.9±0.6	21.4±0.3	32.5±0.1	10.6±0.3
ASA+GSH+H_2O_2	56.3±0.9	41.5±0.4	29.8±0.6	36.4±0.2	14.4±0.1

Table 3

Effect of benzoic acid (BA), ascorbic acid (ASA), reduced glutathione (GSH), and thiourea (Tu) at 10 mM on protein and MDA levels in detached *Cucurbita* leaves treated with 10 mM $CuSO_4$. Protein and MDA levels were determined after 48 h of treatment. Values are means ± S.E.

Treatment	Protein (mg g^{-1} FW)	MDA (nmol g^{-1} FW)
Control	32.7 ± 0.6	70.0 ± 0.3
$CuSO_4$	19.5 ± 0.2	110.2 ± 0.2
BA + $CuSO_4$	23.3 ± 0.4	105.5 ± 0.1
ASA + $CuSO_4$	27.4 ± 0.3	94.3 ± 0.4
GSH + $CuSO_4$	29.6 ± 0.5	73.3 ± 0.7
Tu + $CuSO_4$	32.3 ± 0.2	68.1 ± 0.5

and H_2O_2 treatment. It has been shown that heavy metals can reduce chlorophyll formation by reducing the uptake of Fe^{2+} and Mg^{2+} [34] and by reacting with essential thiol groups in both the protochlorophyllide reductase protein and other enzymes involved in the light dependent synthesis of 5-aminolaevulinic acid [35, 21]. The effects of $CuSO_4$ on the loss of protein and chlorophyll could have resulted from the effects of free radicals produced by treatment with Cu ions.

Fig. 4 shows that MDA level in $CuSO_4$- or H_2O_2-treated detached *Cucurbita* leaves was higher than that of the water-treated controls throughout the entire duration of incubation. This indicates that $CuSO_4$- and H_2O_2-promoted senescence of detached *Cucurbita* leaves is linked to lipid peroxidation. The increase in lipid peroxidation in $CuSO_4$- or H_2O_2-treated detached *Cucurbita* leaves may be a reflection of the decline of antioxidative enzymes. Mazhoudi et al. [25] and Lin and Kao [28] reported a similar increase in lipid peroxidation when plants were treated with either Cu or H_2O_2.

It has been shown that H_2O_2 can react with superoxide to form more reactive hydroxyl radical [8]. Since lipid peroxidation is generally considered to be induced by free radicals [8], it is possible that the effects of $CuSO_4$ or exogenous H_2O_2 on the senescence of detached *Cucurbita* leaves is mediated through them, especially hydroxyl radicals. If this suggestion is correct, then the promotive effect of $CuSO_4$ or exogenous H_2O_2 on the senescence of detached *Cucurbita* leaves should be prevented by the addition of free radical scavengers.

Thus, Tables 3 and 4 show the effect of pretreatment of detached *Cucurbita* leaves with free radical scavengers such as BA, ASA, GSH and TU for 12 h on $CuSO_4$- and H_2O_2 -promoted senescence and lipid peroxidation. It is evident that all tested free radical scavengers reduced senescence caused by $CuSO_4$ or H_2O_2 and at the same time inhibited $CuSO_4$- and H_2O_2-induced lipid peroxidation. Thiourea (TU) was the best scavenger and that is consistent with the report of Lin and Kao [28].

Fig. 5 shows that endogenous H_2O_2 level in $CuSO_4$-treated detached *Cucurbita* leaves was increased compared to the control. On the other hand, the addition of H_2O_2 is expected to result in accumulation of endogenous H_2O_2 in detached *Cucurbita* leaves. Contrary to this expectation, endogenous H_2O_2 did not accumulate in H_2O_2-treated de-

Table 4

Effect of benzoic acid (BA), ascorbic acid (ASA), reduced glutathione (GSH), and thiourea (Tu) at 10 mM on protein and MDA levels in detached *Cucurbita* leaves treated with 10 mM H_2O_2. The enzyme activities were measured after 48 h of treatment. Values are means ± S.E.

Treatment	Protein($mgg^{-1}FW$)	MDA($nmolg^{-1}FW$)
Control	32.4±0.3	69.5±0.4
H_2O_2	17.0±0.3	95.3±0.6
BA+H_2O_2	19.2±0.2	90.6±0.4
ASA+H_2O_2	23.4±0.7	86.4±0.3
GSH+H_2O_2	27.2±0.6	79.3±0.5
Tu+H_2O_2	30.8±0.4	65.7±0.4

tached *Cucurbita* leaves but decreased when compared with water-treated controls.

The fact that addition of H_2O_2 did not result in an accumulation of endogenous H_2O_2 in detached *Cucurbita* leaves can be explained considering that H_2O_2 is being used in metal-catalyzed reactions such as lipid peroxidation. Catalase converts H_2O_2 to oxygen and water. H_2O_2-treatment increased catalase activity (Fig. 1). Since endogenous H_2O_2 level decreased and catalase activity increased, the possibility that the increase in catalase activity is associated with the decrease in endogenous H_2O_2 level in H_2O_2-treated detached *Cucurbita* leaves cannot be excluded. It has been reported that addition of H_2O_2 stimulated the expression of catalase [36]. Furthermore, Willekens et al. [37] clearly demonstrated that catalase can effectively remove exogenous H_2O_2.

The present results suggest that the phytotoxic effects of Cu or exogenous H_2O_2 in detached *Cucurbita* leaves may be caused by an enhanced production of active oxygen species and subsequent high lipid peroxidation.

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