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Redox State in *Solanum* Seedlings under Cadmium Stress Conditions

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Abstract

Cadmium content and distribution as well as its effects on growth and oxidative stress were investigated in 17-day-old tomato seedlings (*Solanum*). The content of Cd increased with external Cd concentrations, and was considerably higher in roots than in shoots. Excess Cd suppressed biomass production of both roots and shoots and reduced chlorophyll content in leaves. Further, substantial increases of H_2O_2 and ascorbate contents, malondialdehyde formation, and antioxidant enzyme activities such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (GPX) were observed in Cd-stressed plants in comparison with controls. The results suggest that the phytotoxic effects of Cd in tomato seedlings may be achieved by an enhanced production of active oxygen species (AOS) and subsequent lipid peroxidation with ascorbate synthesis as singe of tomato tolerance.

Keywords: Cadmium; Oxidative stress; Antioxidant enzymes

Introduction

The high toxicity of excessive metals has been known for a long time. The exposure of plants to metal ions causes growth inhibition or death of plants, coincidental with the alteration of membrane permeability of cells leading to the leakage of ions and pigment destruction [1]. However, the fundamental mechanism of metal phytotoxicity has not yet been characterized, and little is known about the mechanisms related to absorption and phytotoxicity of cadmium (Cd). Active oxygen species (AOS) such as O_2, O_2^-, OH and H_2O_2 are commonly generated under stress conditions [2] and are strong oxidizing species that can rapidly attack all types of biomolecules [3], thus disrupting the normal metabolism of the cell. Meanwhile, generation of AOS, particularly H_2O_2 has been proposed as part of the signaling cascade leading to protection from stresses [4]. For the protection from the oxidative stress, plant cells contain both oxygen radical detoxifying (antioxidant) enzymes such as catalase (CAT), peroxidase (GPX) and superoxide dismutase (SOD), and non-enzymatic antioxidants such as ascorbate, glutathione and α-tocopherol [4]. SOD, the first enzyme in the detoxifying process, catalyzes the dismutation of O_2^- to H_2O_2 and O_2 [5], CAT mediates the cleavage of H_2O_2 evolving O_2, and GPX reduces H_2O_2 to H_2O using several reductants available to the cells [5]. Altered activities of these antioxidant enzymes and antioxidants commonly have been reported in plants, and are used frequently as indicators of stress [6]. In parallel to metal-induced tissue damage or cell death, alteration of both antioxidant enzyme activities [7] and antioxidant levels [5] as well as enhancement of both lipid peroxidation and phytochelatin synthesis [5] have been observed. Therefore, the metal-induced phytotoxicity may be mediated by oxidative stress. However, the changes in AOS metabolism and the enzymes activities involved in scavenging AOS in response of exposing plants to metal have not been investigated in detail. In animals, $HgCl_2$ enhanced lipid peroxidation in several organs, as measured by the thiobarbituric acid reaction for malondialdehyde (MDA), and reduced glutathione level [8], indicating that the oxidative stress-induced lipid peroxidation. The objective of present study is to investigate whether Cd-induced phytotoxicity expressed as growth inhibition and chlorophyll destruction in tomato seedlings is mediated by oxidative stress. The data shows that tomato seedlings exposed to toxic dose of cadmium produce H_2O_2 and the activities of related antioxidant enzymes are altered, indicating that Cd-induced phytotoxicity can be mediated by oxidative stress.

Materials and Methods

Plant material

Seeds of the tomato (*Solanum lycopersicon*. Mill cv 63/5F1) were sterilized in 10 % (v/v) hydrogen peroxide for 20 minutes, and washed abundantly in distilled water afterwards. After imbibition, the seeds were germinated on moistened filter paper at 25°C in the dark. After 7 days, uniform seedlings were transferred to 6 litres plastic beakers filled with continuously aerated, basal nutrient solutions of an initial pH 5.8-6. Plants were grown in a growth chamber. At the age of 10 days after transplant, cadmium was added to the medium as $CdCl_2$ at 0 to 50 µM. After one week of Cd treatment, plants were separated into shoots and roots. Roots were rapidly washed three times with distilled water, and then samples were stored in liquid nitrogen for subsequent analysis or dried at 70°C for at least three days in order to determine both dry material and ionic contents.

Cadmium accumulation

Total shoot and root accumulation of Cd in *Solanum* were determined after 7 days of treatment. Roots and shoots were harvested, washed in deionized water for 2 min, air dried at 80 8C for 2 days, and then ground into a fine powder using a pestle and mortar. The metal concentration in samples was analyzed by atomic absorption spectrophotometry (Philips PYE Unicam PU 9000).

Chlorophyll, pheophytin and malondialdehyde contents

Shoot collected at day 7 after Cd treatment were weighed and ground in 80% acetone. The chlorophyll and pheophytin contents of

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supernatant were estimated according to Arnon [9]. The level of lipid peroxides in the leaves and roots was determined as malondialdehyde (MDA) content by the thiobarbituric acid (TBA) reaction as described by Dhindsa et al. [10].

Measurement of H_2O_2

Content of H_2O_2 in plant tissues was determined based on the modified method of Patterson et al. [11]. H_2O_2 contents were determined by colorimetric method from A_{508}, using H_2O_2 (30% Sigma) (5-50 µM) as a standard.

Enzyme antioxidant assays

All samples were prepared for enzyme analyses by homogenization of the fresh tissue material with a mortar and pestle and a small amount of sand in a solution buffer. The supernatant was used for immediate determination of enzyme activities. All spectrophotometric analyses were conducted on Uvikon 922 spectrophotometer (Kontron Instruments, Italy). Activity of CAT was determined by monitoring the disappearance of H_2O_2 by measuring the decrease in absorbance at 240 nm of a reaction mixture containing 2 ml 29.8 mM H_2O_2 in KPO_4 buffer (pH 7.0) and 1 ml extract [12]. Activity of SOD was assayed by the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) according to the modified method of Becana et al. [13]. Activity of guaiacol peroxidase (GPX) was measured by monitoring the $H_2O_2^-$-dependent oxidation of reduced guaiacol at 470 nm [14]. One unit was defined as the enzymic amount which oxidizes 1 µM guaiacol min^{-1}. Total activities (U) of enzymes were expressed on a fresh weight basis.

Ascorbate

Ascorbate (ASC) and total ascorbate (ASCT) were extracted by grinding fresh leaf tissue in 10% trichloroacetic acid [15]. The assay is based on the reduction of Fe^{3+} to Fe^{2+} by ascorbic acid and formation of a pink colour complex between Fe^{2+} and α α -bipyridyl, with absorption max at 525 nm.

Statistical analysis

The results are the means ± S.E. of at least three independent replicates. The analyses of variance were computed on statistically significant differences determined based on the appropriate *F*-tests. The mean differences were compared utilizing Duncan's multiple range test.

Results

Cd accumulation, seedling growth, pheophytin and chlorophyll levels

The content of Cd in tissues of tomato seedlings increased concurrently with increase in external Cd concentration and exposure time (Figure 1A) ($P < 0.05$). Cd was more accumulated in roots than in upper plant parts; Cd content in roots at 17 days was about 8-fold higher than that in shoots. The effects of Cd on seedling growth, expressed as dry weight and length of shoots and roots, are shown in Figure 1B and Figure 1C, respectively. Cd-exposure induced a substantial depression of both root and shoot dry weights, and this effect varied as a concentration of the exogenous Cd ($P < 0.05$). The growth reduction observed at the high doses of Cd appeared to coincide with an increased accumulation of this metal. However, 10 µM Cd treatments for 17 days was enough to suppress length of shoots and roots, but, the foliar surface was more sensitive at Cd stress than the length. The effects of Cd on chlorophyll *a*, *b*, *total* and pheophytin levels are shown in

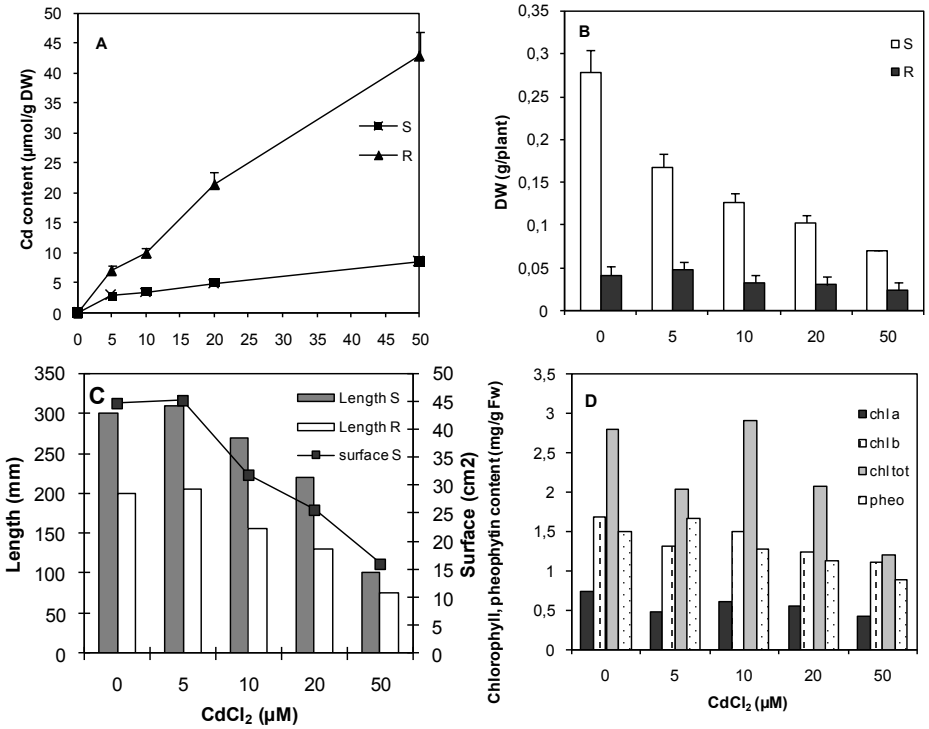

Figure 1: Cadmium accumulation on shoot (S) and root (R) (A). Effect of cadmium stress on shoot and root dry weight (B), on shoot and root length and surface (C) and on chlorophyll *a*, chlorophyll *b*,chlorophyll *total* and pheopphytin content (D) of *Solanum* plants. The plants were grown in presence of 0-50 µM $CdCl_2$ in the medium culture during 17 days. Values are the means ± SE of triplicates from five independent experiments.

Figure 1D. With a substantial amount of Cd accumulation (Figure 1A), Cd-exposure for 7 days was enough to decrease chlorophyll content particularly the chlorophyll *b* and total. However, cholohyll *a* and pheophytin were more resistant.

H_2O_2 production and lipid peroxidation (MDA)

H_2O_2 content in roots was much higher than in shoots (Figure 2A). Subjecting tomato seedlings to up to 50 µM Cd for 17 days increased the level of endogenous H_2O_2 in comparison with control plants, and the effect of Cd on the H_2O_2 level measured at day-17 was much higher in shoots than in roots. The increase of H_2O_2 level in shoots was 9 fold than in roots. To know whether lipid peroxidation was involved in the reduction of both seedling growth levels with Cd treatments, MDA formation was investigated (Figure 2B) ($P < 0.05$). A consistent increase in MDA level paralleled to the H_2O_2 level observed at day-17.

Antioxidant enzymes

The activities of SOD, CAT and GPX were investigated to determine whether Cd-exposure influenced these antioxidant enzymes (Figure 3). All enzyme activities, estimated on a fresh weight basis, were substantially increased by Hg-exposure, depending on exposure time and treatment levels. Compared to the controls, the activity of SOD markedly increased in both leaves and roots exposed to Cd (Figure 3A) ($P < 0.05$). Seven-day exposure to 10 µM Cd was enough to increase the activity, and the increased SOD activities paralleled the levels of H_2O_2 formed in shoots and roots (Figure 3). Examination of two enzymes,

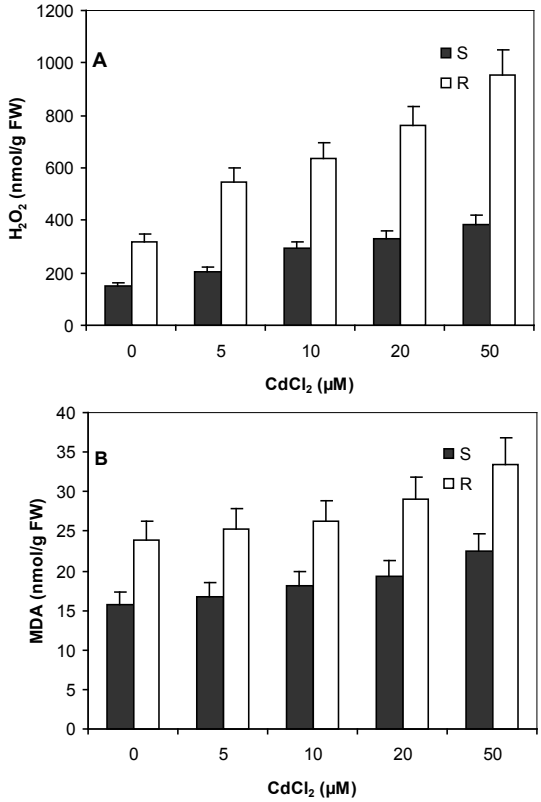

Figure 2: Effect of cadmium stress on shoot (S) and root (R) H_2O_2 content (A), on shoot and root malanoaldehyde (MDA) content (B) of *Solanum* plants. The plants were grown in presence of 0-50 µM $CdCl_2$ in medium culture during 17 days. Values are the means ± SE of triplicates from five independent experiments.

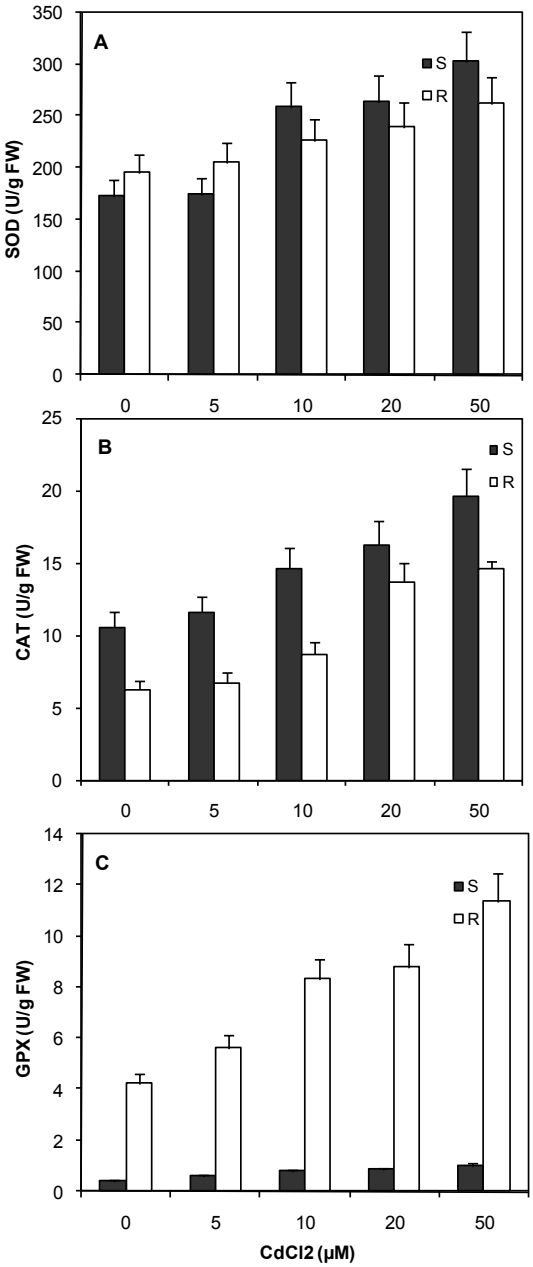

Figure 3: Effect of cadmium stress on shoot (S) and root (R) superoxide dismutase activity (SOD) (A), on shoot and root catalase acyivity (CAT) (B) and on shoot and root guaicol peroxidase activity (GPX) of *Solanum lycopersicum* plants. The plants were grown in presence of 0-50 µM of $CdCl_2$ in the culture medium during 17 days. Values are the means ± SE of triplicates from five independent experiments.

which decompose the H_2O_2 generated by SOD, indicated that the activities of CAT and GPX also increased in response to Cd exposure. The CAT activity (Figure 3B) in the roots was not changed with 5 µM Cd but increased with 10 µM Cd compared to the controls. Meanwhile, when subjected to Cd stress for up to 50 µM, roots maintained higher levels of activity compared to the controls ($P < 0.05$). The levels of H_2O_2 formed in response to Cd-exposure (Figure 2A) might be comparable to the activities of CAT particularly at the highest dose of Cd. The unexpected low H_2O_2 levels especially in the shoots measured at day-

17 with an increased SOD activity might be due to the increased CAT activity. Mean GPX (Figure 3C) activity was higher in roots than in leaves. In shoots, treatment with 50 µM Cd for 17 days resulted in a marked increase in GPX activity ($P < 0.05$). In roots, the results also indicated that the lowered GPX activity measured was recovered at 50 µM, and the enhanced GPX activity might contribute to the reduction of H_2O_2 level measured in shoots and roots.

Ascorbate and total ascorbate

Changes in ascorbate and total ascorbate content were more important in roots than in shoots (Figure 4A and B). ASC and ASCT concentrations showed a significant increase ($P < 0.05$). The effect of excess Cd was particularly marked in shoots than in roots of tomato plants.

Discussion

Although a number of studies have demonstrated that metals are generally immobilized to a far greater extent at the site of metal uptake [16], details have not been provided with respect to time and concentration in specific tissues to allow for distribution in the growing plant. Since translocation will require the movement of Cd across the endodermis, membrane integrity to allow the symplastic movement might be important for the continuous Cd accumulation in shoots. High Cd accumulation in roots (Figure 1A) in spite of high MDA production (Figure 2B) indicates the extent of cell damage which might be explained on this basis. The lowest accumulation in the shoots also implies that absorbed Cd is not readily mobilized and redistributed in the plants. The observed changes in the biomass of tomato seedlings were consistent with previous results obtained at high Cd in pea [17] and tobacco [18]. The growth reduction observed at the levels of Cd in treatments (Figure 1) closely coincided with a considerable accumulation of this metal, especially in the roots. The growth reduction might be due to both the reduction in chlorophyll contents in leaves (Figure 1D) and membrane damage indicated as an enhanced lipid peroxidation resulted by MDA accumulation (Figure 2B). It has also been suggested that heavy metals induce the deficiency in nutrients by reducing the uptake and transport of some mineral nutrients since metal accumulation in root may block the entry or binding of the ions such as Ca, Mg and K to ion-carriers [19]. The reduction of chlorophyll content observed in this study might be due to an increased cell or tissue damage estimated by MDA production. Destruction of lipid components of membrane by lipid peroxidation causes membrane impairment and leakage. Meanwhile, it has also been suggested that the reduction in chlorophyll content in the presence of metal is caused by an inhibition of chlorophyll biosynthesis [20]. The present study clearly indicates that Cd-exposure results in an increase in H_2O_2 content in plants (Figure 2A). Although the mechanism of Cd-induced H_2O_2 formation is not presently known, heavy metals are known to be involved in many ways in production of AOS [21]. The H_2O_2 accumulation after Cd-exposure may be produced in a manner similar to H_2O_2 in plants cold-stressed. It is conceivable that a decrease of enzymic and non-enzymic free radical scavengers caused by heavy metals may also contribute to the shift in the balance of free radical metabolism towards H_2O_2 accumulation, and H_2O_2 and O_2^- may interact in the presence of certain metal ions or metal chelates to produce the highly reactive hydroxyl radical (OH). The increased H_2O_2 and OH production might be involved in the lipid peroxidation observed in tomato seedlings. The susceptibility to oxidative stress is a function of the overall balance between the factors that increase oxidant generation and those substances that exhibit antioxidant capability [22]. Some protective enzymes are activated in plants when production of oxygen free radicals is stimulated by stresses, and increased SOD activity may be considered as circumstantial evidence for enhanced production of AOS [23]. The enhanced SOD activity observed in this study (Figure 3A) might support the hypothesis that the H_2O_2 resulted from oxygen free radicals including O_2^-. The increased CAT activity (Figure 3B) might be related to the lowered H_2O_2 production observed in figure 2, and indicated that the role of CAT might be critical to removal of H_2O_2 induced by Cd. Although Cd inhibits CAT activity, the enzyme can take part in an efficient defence mechanism against Cd-induced oxidative stress in tomato bean. Because of a significant increase in GPX activity and strong qualitative metal-specific changes in the GPX isozyme pattern, the role of GPX in removal of H_2O_2 might be critical in metal-induced oxidative stress. The activity of GPX was not highly changed in the shoots, but was lowly increased in 50 µM of Cd. It might be possible that Cd-induced GPX activity is associated with cell wall lignification and, consequently, with a decrease of root growth (Figure 1B). GPX has been postulated to stiffen the cell wall and GPX-mediated lignification decreases the cell wall plasticity, and therefore reduces cell elongation, which might represent a mechanical adaptation to stress conditions [24]. Based on the present work, it can be concluded that the amount of Cd in the tissues of tomato seedlings might be associated with the reduction of both biomass and chlorophyll. Toxic concentrations of Cd cause oxidative stress, as evidenced by the

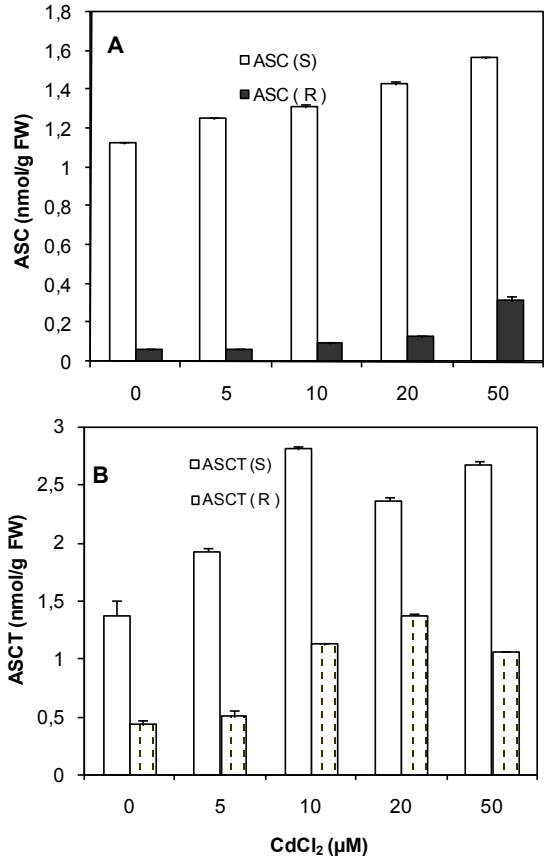

Figure 4: Effect of cadmium stress on shoot (S) and root (R) ascobate content (A) and on shoot and root total ascorbate content (B) of *Solanum lycopersicon* plants. The plants were grown presence of 0-50 µM $CdCl_2$ in the culture medium during 17 days. Values are the means ± SE of triplicates from five independent experiments.

increased H_2O_2 formation and lipid peroxidation in shoots and roots of seedlings. The reduction of both biomass and chlorophyll concentration might result from lipid peroxidation-mediated cell damage in tissues. Cd-induced H_2O_2 formation may be associated with an increased activity of SOD for O_2^- conversion. Although parallel increases in activities of CAT and GPX occur and might contribute to lower H_2O_2 content, the antioxidant potential in the tissues of seedlings might not be enough to block the lipid peroxidation process. The high GPX activity might contribute to suppress elongation of both shoots and roots. Summing up, it was proposed that the reduced growth of tomato seedlings exposed to toxic levels of Cd may be induced by an enhanced production of toxic oxygen species and subsequent lipid peroxidation (Figure 5). The present results of ascorbate and total ascorbate contents emphasize its roles in plant stress tolerance (Figure 4). Ascorbate is also considered crucial in scavenging AOS, particularly those arising from exposure to heavy metal pollutants such as cadmium. Summarising our results it can be concluded that the heavy metal stress caused typical biochemical changes in wheat seedlings concerning contents of AOS, ascorbate and antioxidant enzymes. The differences between the two organs are due to the different transport processes and their different biological pathway. Ascorbate synthesis seemed to reduce the negative effects of cadmium toxicity in tomato seedlings. Further research is needed to find out the relationship between the cadmium stress and these biochemical changes in tomato seedlings and also to prove the beneficial role of ascorbate. Although the antioxidant role of ascorbic acid is fundamental, this vitamin can be involved in other chemical reactions of cellular metabolism. The effect of ascorbate on the cell may be either protective or toxic.

Our results indicate that GPX is the highly sensitive site of antioxidant enzymes under Cd stress. The rapid activation of GPX under Cd stress is mainly due to the increase of ASC concentration at the site where GPXs are compartmentalized, which makes the antioxidant efficiency not sufficient to scavenge ROS, resulting in the accumulation of ROS-like H_2O_2. H_2O_2 is active to interact with thiol groups in proteins, especially to that in Rubisco protein and oxidized them. These results may contribute to clarifying the mechanism of oxidative stress and its physiological consequence under Cd stress, and help to find ways to enhance plant resistance to Cd stress.

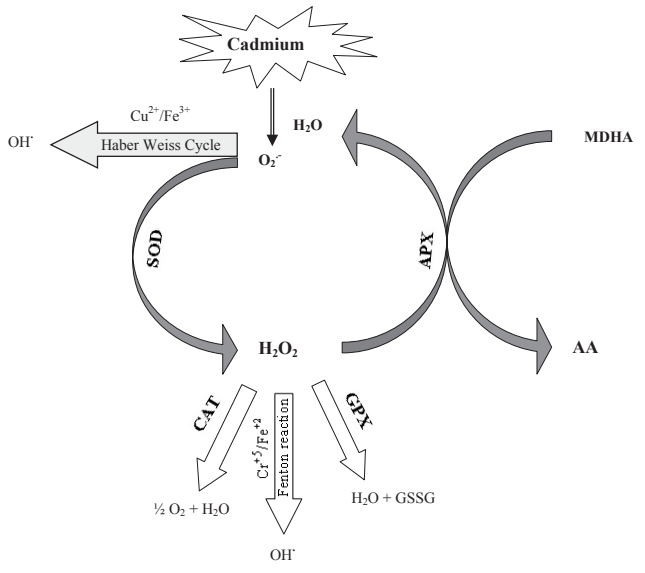

Figure 5: Mechanism of active detoxification with antioxidant enzymes in *Solanum lycopersicon*.

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