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OXIDATIVE STRESS AND DNA DAMAGE IN BROAD BEAN (*VICIA FABA* L.) SEEDLINGS INDUCED BY THALLIUM

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Abstract–Thallium is a metal of great toxicological concern as it is highly toxic to all living organisms through yet poorly understood mechanisms. Since thallium is accumulated by important crops, the present study aimed to analyze the biological effects induced by bioaccumulation of thallium in broad bean (*Vicia faba* L.) as well as plant's antioxidative defense mechanisms usually activated by heavy metals. Thallium toxicity has been related to production of reactive oxygen species in leaves and roots of broad bean seedlings following short-term (72 h) exposure to thallium (I) acetate (0, 0.5, 1, 5 and 10 mg/L) by evaluating DNA damage and oxidative stress parameters as well as antioxidative response. Possible antagonistic effect of potassium was tested by combined treatment – 5 mg/L of thallium (Tl+) and 10 mg/L of potassium (K+) acetate. Accumulation of Tl+ in roots was 50 to 250 times higher than in broad bean shoots and accompanied by increase in dry weight and proline. Despite responsive antioxidative defense (increased activities of superoxide dismutase, ascorbate peroxidase and pyrogallol peroxidase), Tl+ caused oxidative damage to lipids and proteins as evaluated by malondialdehyde and carbonyl group levels and induced DNA strand breaks. Combined treatment caused no oxidative alternations to lipids and proteins though it induced DNA damage. The difference in Tl-induced genotoxicity following both acellular and cellular exposure implies indirect DNA damage. Results obtained indicate that oxidative stress is involved in the mechanism of thallium toxicity and that the tolerance of broad bean to thallium is achieved, at least in part, through the increased activity of antioxidant enzymes.

Keywords–Thallium, Plant, Proline, Antioxidant, Genotoxicity

INTRODUCTION

As most abiotic and biotic stress, heavy-metal stress in all living organisms often results in the production of reactive oxygen species (ROS) which are relatively reactive compared to molecular oxygen and thus potentially toxic [1-3]. Consequent leaking of electrons from photosynthetic and mitochondrial electron transport chains to molecular oxygen enables higher production of ROS such as singlet oxygen, superoxide, hydrogen peroxide and hydroxyl radicals which can also be formed in peroxisomes and plasma membranes. These cytotoxic ROS can disturb normal metabolic processes through oxidative damage of lipids, proteins and DNA. However, ROS exert negative effects only if present in high quantities. Otherwise, many important roles have been attributed to ROS such as tracheary element formation, lignification, signalling and regulation of antioxidative gene expression [3]. A regulated balance between oxygen radical production and destruction is achieved by plant antioxidative system that includes enzymatic molecules such as superoxide dismutase ([SOD], enzyme classification (EC) number ~~(EC)~~ 1.15.1.1)), ascorbate peroxidase ([APX], EC 1.11.1.11), non-specific peroxidases ([POX], EC 1.11.1.7) and catalases ([CAT], EC 1.11.1.6) and various antioxidants of low molecular mass. Tolerance to heavy metal stress has been correlated with efficient antioxidative defense system, as shown by many authors [3-6]. Much information is available on the effect of redox heavy metals on various antioxidant processes in plants [2, 6, 7]. In contrast to redox, non-redox metals (cadmium, mercury, thallium, zinc) are unable to perform single electron transfer reactions and do not produce ROS directly, but generate oxidative stress by interfering with the antioxidant defense system [1, 5, 8].

Thallium is a highly toxic metal that plays no role in the metabolism of plants or animals [9-11]. Although widely distributed in the environment, thallium is present in the earth's crust at

very low concentrations (between 0. 3 and 0. 7 mg/kg). However, prevalence of thallium in the natural environment has steadily increased (more than 1500 tons of thallium per year are released into the environment) as it is a by-product of the refining process of iron and zinc in smelting plants [11, 12]. Furthermore, since the use of thallium in the industry has substantially increased in the last decades, it has attracted greater attention as a potential pollutant on a large scale [11]. So far, several major contaminations of food chain and consequent hazard to animal and human health have been described [12-14]. Yet, the mechanisms of thallotoxicity are not entirely understood. Villaverde and Verstraeten [15] proposed a potential mechanism of damage to biological membranes which includes interaction of monovalent thallium (Tl+) with membrane phospholipids and increase in the membrane permeability. Findings of Galván-Arzate et al. [16] have demonstrated that thallium toxicity is closely related to increased ROS formation in animal tissues. Regarding plant systems, several studies deal with bioaccumulation of Tl+ in plants [13, 17-20], but effects of Tl+ on the plant's stress defense mechanisms, including antioxidative ones, have not been studied so far. Except for the obvious reason of high toxicity, it is possibly due to the fact that it is often undetected by classical analytical methods and that, until recently it was still used as a depilator or as a rodenticide and insecticide in many countries. It is brought to agricultural soils as a trace compound of sludge from water treatment and in potassium fertilizers, the maximum admissible level of thallium in agricultural soil being 1 mg/kg dry weight [11]. The major pathway of exposure for humans is the ingestion of vegetables grown in thallium contaminated soils and even small doses (8 mg/kg) can be lethal to man [9]. In the environment, this heavy metal exists in its oxidized states as monovalent Tl+ and trivalent Tl3+ species [21, 22]. Thallium can be readily taken up by plants because it is generally present in the soil as thermodynamically stable Tl+ and as such is an analogue of potassium [22].

Consequently, Tl+ interferes with the vital pottasium-dependent processes. It substitutes K+ in the activation of several cation activated enzymes such as (Na^+/K^+)-adenosine triphosphatase and shows high affinity for sulfhydril groups from proteins and other molecules. The capability of Tl+ to mimic K+ in metabolic processes has been attributed to the remarkable inability of cell membranes to distinguish between Tl and K, possibly due to their similar ionic charges and radii [9, 11, 23].

Beside well-known oxidative stress indicators such as carbonyl and malondialdehyde levels, heat-shock protein of 70 kDa (HSP70) was used in the present work as a biomarker of heavy-metal produced stress [24, 25]. On the other hand, proline accumulation has been proposed to play an important role in ameliorating environmental stress in plants, including heavy-metal stress, acting as an osmoprotectant, a protein stabilizer, a metal chelator and an antioxidant [26].

The study was conducted under controlled experimental conditions using the aqueous solution of Tl+ rather than Tl+ contaminated soil in order to evaluate the exclusive effect of Tl+ as it is highly soluble in water and has a very low stability constant with both organic and inorganic ligands [22, 27]. It has been stated by many studies that higher plants can be used as monitors of environmental pollutants and that results obtained could be extrapolated to higher animals since they are both eukaryotic organisms [28-30]. Also, plants are less expensive and less time consuming than mammalian systems. Broad bean (*Vicia fabia* L.) was chosen as an object of the study as it is a widely spread crop plant and is frequently used as a model plant. Based on the concentrations of thallium in natural environment [9, 11] as well as on preliminary results, broad bean seedlings were exposed to two lower Tl+ treatments (0.5 and 1 mg/L). To evaluate the capacity of the capacity of the tolerance mechanisms of plants to possible mechanisms to higher Tl-contamination in the environment, seedlings were also exposed to 5 and 10 mg/L Tl+.

The objective of the present study was to investigate whether short-term exposure of broad bean to Tl+ can induce direct DNA damage and produce significant changes in malondialdehyde (MDA) and carbonyl groups (C=O) levels in correlation with alternations in endogenous antioxidant system. In addition, combined treatment (Tl+K) was applied on broad bean seedlings in order to evaluate possible antagonistic effect of K+ against Tl+ toxicity.

MATERIALS AND METHODS

Plant material and stress conditions

Vicia fabia L. seeds (var. aquadulce) were irrigated by distilled water for 24 h, placed on moist filter paper in Petri dishes for several days and then transferred to glass containers filled with distilled water. The plantlets were grown for 10 d under a 16-h-photoperiod of fluorescent light (90 $\mu E/m^2s^1$) at 26±2 °C. Exposure assays were carried out at 0.5, 1, 5, and 10 mg/L of Tl(I) acetate (Sigma-Aldrich, St.Louis, MO, USA). Rooted seedlings were also exposed to combined treatment – 5 mg/L of Tl-acetate and 10 mg/L of K-acetate (Tl+K) – and to 10 mg/L of K-acetate only (acetate control) to evaluate the effect of acetate ion. Acetic salt of Tl+ was chosen because it was found to be the least toxic compared to nitrate or chloride salts [21]. Control plants were kept in distilled water during the entire assay. Samples were taken after 72 h of exposure.

Analytical methods

Broad bean roots and shoots, were separated, weighed (fresh wt) and oven-dried (dry wt) at 80°C for 48 h by which time constant dry weights were obtained. Relative water content was determined according to calculation: fresh weight (g)-dry weight (g)/fresh weight (g).

The Tl and K contents in the roots and shoots of broad bean seedlings were determined by graphite furnace atomic absorption spectrophotometer (PerkinElmer AA 600; Waltham, MA, USA) and flame atomic absorption spectrophotometer (PerkinElmer AA 300,) respectively, after microwave wet digestion (Anton Paar Multiwave 3000, Graz, Austria, EU) of the dried and powdered material in 10 ml of supra-pure concentrated HNO_3 at 230°C. Estimation was carried out in triplicate.

Free proline content was measured by the method of Bates et al. [31] using the ninhydrin reagent. Proline concentration was read at 520 nm and determined from calibration curve using L-Proline (Sigma-Aldrich) as standard and expressed as nmol proline/g fresh weight.

The alkaline version of both cellular (in vivo) and acelular (in vitro) comet assay has been done according to Gichner et al. [32] with slight modification (20 min denaturation, 15 min electrophoresis at 1 V/cm, 300 mA). In the acellular comet assay the procedure was the same as in cellular version except the leaf nuclei were exposed to a stressor after isolation and agarose embedment. In the cellular comet assay nuclei were isolated from fresh leaves and root tips previously frozen in liquid nitrogen. For each of three replicas, 50 randomly chosen cells were analyzed with a fluorescence microscope Zeiss Axioplane with an excitation filter BP 520/09 nm and a barrier filter of 610 nm. A computerized image analysis system (Komet version 5, Kinetic Imaging, Liverpool, UK) was employed.

Lipid peroxidation was determined by estimating the amount of MDA content using the thiobarbituric acid method described by Heath and Packer [33]. The MDA content was calculated from the absorbance at 532 nm by using extinction coefficient of 155 ml/mmol cm. Hydrogen peroxide was estimated according to the method of Mukherjee and Choudhuri [34] using the titanyl-sulphate (Sigma-Aldrich) and concentrated NH_4OH solution. The absorbances

of the dissolved peroxide-titanium were read at 415 nm and H_2O_2 contents calculated from a standard curve and expressed as μmol H_2O_2 /g fresh weight. The amount of protein oxidation was estimated by the reaction of carbonyl groups with 2, 4-dinitrophenylhydrazine (Sigma-Aldrich), as described in Levine et al. [35]. After 2, 4-dinitrophenylhydrazine-reaction, the carbonyl content was calculated by absorbance at 370 nm, using an extinction coefficient for aliphatic hydrazones of (22 ml/mmol cm) and expressed as nmol carbonyl/mg protein.

Analysis of antioxidant enzyme acitivities: Shoot or root tissue was homogenized in 50 mM KPO_4 buffer (pH 7) including 1 mM ethylene diamine tetraacetic acid (Sigma-Aldrich) and polyvinylpolypyrrolidone (Sigma-Aldrich). The homogenates were centrifuged (Sigma 3K18 Centrifuge, Germany, EU) at 25,000 *g* for 30 min at 4 °C and supernatants used for enzyme activity and protein content assays. Total soluble protein contents of the enzyme extracts were estimated according to Bradford [36] using bovine albumine serum (Sigma-Aldrich) as standard.

The activity of superoxide dismutase (SOD) was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (Sigma-Aldrich) following the method of Beauchamp and Fridovich [37]. One unit of SOD was taken as the volume of the enzyme extract causing 50% inhibition of nitroblue tetrazolium reduction. Ascorbate peroxidase (APX) activity was done according to Nakano and Asada [38]. The ascorbate oxidation was followed at 290 nm and its concentration calculated using the molar extinction coefficient 2.8 ml/mmol cm. One enzyme unit was defined as μmol oxidized ascorbate per min. The activity of non-specific peroxidase (POX) was measured using pyrogallol (Sigma-Aldrich) as substrate according to Chance and Maehly [39]. The formation of purpurogallin was followed at 430 nm and was quantified taking into account its extinction coefficient (2.47 ml/mmol cm). Catalase (CAT) activity was determined by the decomposition of H_2O_2 and was measured spectrophotometrically

by following the decrease in absorbance at 240 nm [40]. Activity was calculated using the extinction coefficient (0.04 ml/mmol cm) and μmol H_2O_2 decomposed per min was defined as unit of CAT.

The specific enzyme activity for all enzymes was expressed as units/mg protein.

Analysis of HSP70: Shoot/root samples were homogenized in Tris-HCl extraction buffer pH 8 containing 17.1% (w/v) sucrose, 0.1% (w/v) ascorbic acid, 0.1% (w/v) cysteine-hydrochloride (Sigma-Aldrich) with addition of polyvinylpolypyrrolidone and then centrifuged at 25,000 *g* for 30 min. Total protein concentration in the supernatant was determined using bovine albumine serum as standard. Aliquots of each homogenate were mixed with corresponding volumes of denaturating 0.065 M Tris-HCl buffer containing 6% (w/v) sodium dodecyl sulphate (SDS, Sigma-Aldrich), 6% (v/v) β-mercaptoethanol (Sigma-Aldrich), 30% (v/v) glycerol and 0.01% (w/v) of bromphenol blue. The extracts were boiled for 2 min. Constant protein weights 9 or 12 μg of total protein per lane were analyzed by SDS-polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, USA) and subsequent Western blotting at 60 V (Bio-Rad). The resolving gel was made at 10% of polyacrylamide (w/v). Standard proteins of known molecular weights were run in the same gel (Fermentas, Glen Burnie, MD, USA). The membranes were blocked with 10% (w/v) non-fat powdered milk solution made in Phosphate buffered saline (58 mM Na_2HPO_4, 17 mM NaH_2PO_4, 68 mM NaCl) pH7.4 containing 1% (v/v) of Tween 20 (Sigma-Aldrich) and incubated with a rabbit monoclonal antibody raised against the pea HSP70 (diluted 1:1,000) overnight at 4°C. The secondary antibody was an alkalinephosphatase-anti-rabbit IgG (Sigma-Aldrich) diluted 1:2,000. The membranes were developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich).

Statistical analysis

For each analysis, data were compared by analysis of variance (ANOVA), using STATISTICA 7.1 (StatSoft, Tulsa, OK, USA) software package, and differences between corresponding controls and exposure treatment were considered as statistically significant at $P < 0.05$. Each data point is the average of six replicates unless stated otherwise.

RESULTS

Uptake of Tl and K and their effect of on water status and dry matter

Thallium accumulated in *V. faba* shoots and roots, but the accumulation in roots was 50 to 250 times greater than in shoots (**Table 1**). Massive accumulation of Tl in roots was accompanied with partial maceration of the tissue especially on the tips, but only in response to higher Tl concentrations. However, the phytotoxic effect has not been observed under the combined treatment. Thallium content in both roots and shoots showed almost linear increase with increasing Tl concentrations, the deviation from that observed only under 10 mg/L in shoots. Contrary to that, K content decreased in broad bean plants exposed to Tl except under combined treatment (5 mg/L of Tl+10 mg/L of K) where it was either similar to control (shoot) or even higher (roots). However, regardless of two times higher K concentration in Tl+K treatment, Tl accumulated to the same level as in treatment of the same Tl concentration (5 mg/L Tl). Shoot and root dry matter and water status showed opposite patterns of change: with increase in dry matter, decrease in water status was observed. Although found significant, shoot and especially root water status was not seriously affected by Tl treatments – under highest Tl concentration it was decreased by only 4% compared to control. Shoot dry biomass decreased under lowest Tl treatment for 33% while highest increase of dry biomass in shoots and roots under Tl treatments amounted to 27%. However, the visual symptoms of Tl toxicity were not observed in broad bean leaves.

Effect of Tl on proline content

Constitutive levels of proline which is expected to serve not only as compatible osmolyte but also as ROS scavenger were 3 to 4 times greater in broad bean shoots than in roots (**Fig. 1**). In addition, proline level in shoots was also less affected by Tl toxicity compared to that in roots. Contrary to shoot, proline accumulated in roots with growing Tl concentrations. During experiment, acetate control showed proline levels similar to control.

Effect of Tl on H_2O_2, malondialdehyde and carbonyl groups levels

Malondialdehyde (MDA), an indicator of the extent of lipid peroxidative reactions to membranes, and H_2O_2 levels showed good correlation in both roots and shoots (**Fig. 2**A, B). Both parameters increased under higher Tl treatments but MDA level showed marked increase under 1 mg/L also. Thallium induced oxidative damage to proteins which was evaluated by content of carbonyl groups (Fig. 2C). The level of carbonyl groups in both shoots and roots increased under Tl toxicity. The increase in shoots ranged from 19 to maximum 37% while those in roots from 25 to approximately 100% (at 10 mg/L) of the control values. Combined treatment caused no changes in the level of oxidative stress indicators after 72-h period.

Effect of Tl on DNA

Potential genotoxic effect of Tl was checked by cellular comet assay. Thallium induced DNA damage to much higher extent in broad bean shoots than roots (**Table 2**). To determine the direct effect of monovalent thallium on DNA, the acellular comet assay, in which DNA is no longer held under the constraints of any metabolic pathway or cell wall barriers, was applied. However, treatments of the isolated shoot nuclei did not result in significant increase of average median tail moment. The direct DNA damage induced by increasing H_2O_2 concentrations (positive control) linearly increased the values of average median tail moment from 0.63±0.08 to 45.9±0.44.

Effect of Tl on antioxidant enzyme activities

Activity of SOD, the first antioxidative enzyme in line, increased in response to Tl toxicity. However, the increase was more prominent in roots and almost dose-response (**Fig. 3**A). Despite additional K, Tl+K treatment also induced SOD activity in both plant organs.

Excessive levels of H_2O_2 could be minimized through the activities of catalase and peroxidases. Ascorbate peroxidase (APX) activity significantly increased under higher Tl and Tl+K treatments in shoots and under all Tl concentrations, including combined treatment, in broad bean roots (Fig. 3B). Under both control as well as Tl treatments shoots maintained higher APX activity than roots. Opposite to APX, POX activity was generally higher in roots than in shoots (Fig. 3C). Only the highest Tl treatment increased POX activity in shoots while POX activity in roots was elevated under all Tl treatments. Higher Tl treatments significantly decreased catalase (CAT) activity (Fig. 3D) in roots while combined treatment (Tl+K) increased CAT activity in both plant organs. Acetate control had no significant effect on the activities of antioxidative enzymes.

Effect of Tl on HSP70 expression

Two isoforms of heat-shock protein of 70 kDa (HSP70) were found in the untreated as well as in Tl- and K-acetate treated leaves after 72 h (**Fig. 4**). Both isoforms accumulated less with increasing Tl concentrations. Out of three HSP70 isoforms present in roots, isoform HSP70 3 accumulated more in response to 0.5, 1, 5 mg/L Tl and combined treatment compared to control while the intensity of all three bands decreased under highest Tl treatment.

DISCUSSION

Thallium is a metal that has not been studied extensively although it is one of the most toxic environmental and industrial pollutants. With respect to degree of toxicity, Tl(I) ranks alongside

Pb, Hg, and Cd [9-11, 23]. Even though under natural conditions thallium occurs relatively rarely, human activities such as mining and ore processing cause increase of the element in the environment. Several studies showed that, due to high uptake of Tl by crops, Tl can be transferred from soils to crops and remarkably concentrate in food crops thus increasing possible severe impacts on human health [13, 20]. A positive correlation between plant uptake and Tl concentration in the solution has also been demonstrated in laboratory studies using nutrient solutions [41]. Our present study is in agreement with those since broad bean accumulated Tl in dose-dependent way, though with considerably higher levels in the roots. In the study of Pötsch and Austenfeld [19], the growth of broad bean was not affected by thallium nitrate despite high accumulation of thallium within the roots. The authors suggested that broad bean plants tolerate high Tl concentrations by limiting the amount of the transported metal to the leaves. In the present study, thallium content in broad bean roots was 147 to 350 times higher than the Tl+ supplied in tested solutions (Table 1). Studying the uptake of Tl by vegetables grown in thallium-rich soil, LaCoste et al. [18] also found that due to predominant root accumulation only green bean and tomato would be safe for human consumption after exposure to Tl treatments between 0.7 and 3.7 mg/kg. Exceptions to the rule were mostly plants from *Brassicaceae* family, e.g., radish, green cabbage and rape and well-known Tl hyperaccumulator *Iberis intermedia*. In those plants tolerance to relatively high concentrations of thallium seems to be a result of complexation in plant leaves [27]. The pattern of heavy metal distribution in favor of roots versus shoots is not unusual [7, 20]. It was found that different root tissues act as barriers to apoplastic and symplastic Cd and Pb transports and hence their translocation to shoot gets restricted [42].

As previously reported in studies done on animal tissues, Tl+ acts as K+ anatagonist and can substitute K+ in many physiological reactions and consequently disturb K uptake [23].

Absorption of thallium by plants was found to be under metabolic regulation using the transport mechanisms for K; that is K acts as a non-competitive inhibitor [43]. Thallium negatively influenced endogenous K content in broad bean seedlings but exogenously added K succeeded to maintain shoot and root potassium contents of broad bean seedlings (Table 1). These findings corroborate results of Siegel and Siegel [43] who, studying the growth of cucumber seedlings, also observed the positive effect of exogenously added K on Tl toxicity. However, regardless of the application of two times higher exogenous K, the accumulation of Tl in broad bean seedlings under combined treatment equaled that in seedlings treated with 5 mg/L Tl only.

It has been shown in different plant species that heavy metals decrease water status and concentrations of photosynthetic pigments which eventually results in wilting and growth reduction [44]. Thallium also affected water status of broad bean roots, which was accompanied by concomitant increase of biomass accumulation as well as proline content with increasing Tl concentrations. Proline content and water status were less disturbed in broad bean shoots, probably due to lesser Tl accumulation. Increase of proline as a function of metal accumulation was observed under heavy-metal toxicity [45, 46]. A far greater proline rise is known to occur due to water deficit [47]. Thus, it is likely that proline accumulation in heavy-metal treated plants is not related to osmotic adjustment but to the ROS detoxification or to the enzymes protection through the chelation of metal ions [48]. Our results corroborate the role of proline as an efficient ROS scavenger only up to 1 mg/L of Tl (Fig. 2). On the other hand, our study revealed positive correlation between enzyme activities (SOD, APX, and POX) and proline accumulation in roots under Tl treatments (Fig. 4), thus suggesting that proline might exert protective effect due to formation of a metal-proline complex [49].

Predominant accumulation of Tl in roots may also present a protective strategy for photosynthetic pigments from Tl-induced oxidative damage as no effect on chlorophylls and carotenoids contents were observed following 72-h exposure to monovalent thallium (data not shown).

Heavy metal toxicity is considered to induce greater production of ROS which may result in significant alternations in cell structure and mutagenesis. Thallium induced oxidative stress in broad bean seedlings as evident from the increased endogenous H_2O_2, malondialdehyde and carbonyl groups contents as well as DNA damage (Table 2). However, absence of DNA damage observed in the acellular Comet assay and dose-response DNA-damage induced by hydrogen peroxide in broad bean nuclei as well as increased contents of endogenous H_2O_2 (Fig. 2A) lead to conclusion that Tl-induced DNA damage is preferentially mediated indirectly via ROS metabolic products. The studies of Cd genotoxicity assessed by comet assay with transgenic catalase-deficient tobacco [32] or micronucleus assay with broad-bean [50, 51] support this interpretation. Although no data until now existed on Tl-induced oxidative stress in plant tissues, several studies with animal tissues have already related the effects of Tl+ to oxidative stress promotion [16, 52, 53]. In the latter study, it was found that Tl+ significantly increased mitochondrial H_2O_2 levels and ROS content in rat adrenal cells thus impairing mitochondrial functioning and leading to cell damage and death. Thallous cations (Tl+) affected the functionality of isolated mitochondria through the opening of transition pores, causing the uncoupling of the respiratory chain [54]. As Tl is not a redox metal like Cu or Fe, it cannot catalyse Fenton-type reactions yielding hydroxyl radicals, but it may cause oxidative stress in plants by disturbing the photosynthetic electron transport that will lead to greater production of ROS. In the present study, Tl+ increased H_2O_2, and caused oxidative alternations to lipids and proteins as seen by increased malondialdehyde

and carbonyl groups levels of both broad bean shoot and root. The greater extent of lipid peroxidation could be explained by the fact that the process itself gets initiated not only by hydroxyl radical formed from H_2O_2, but also by superoxide radical, singlet oxygen or by lipooxygenases [55]. Proteins can be affected by different ROS either directly by oxidation of amino acid side chains or by secondary reactions with aldehydic products of lipid peroxidation or glycosylation which gives rise to the production of carbonyl-groups in the protein molecule [56]. The significant increase in the levels of SOD, APX, and POX activities in broad bean seedlings at given Tl concentrations and duration demonstrate efficient antioxidative defence against Tl-induced oxidative stress (Fig. 3). Lipid peroxidation and H_2O_2 levels, SOD, APX, and CAT activities increased in pea roots and leaves exposed to similar concentrations of cadmium as well [5]. Within a cell, SOD constitutes the first line of defence against ROS. Superoxide dismutase catalyses the dismutation of superoxide to H_2O_2 and O_2. Thus, an increase in SOD activity indicates higher production of endogenous H_2O_2 levels which has been seen in the case of Tl treatment, especially under higher concentrations. The induction of APX and POX activities coincided with increased levels of H_2O_2 at higher Tl treatments in both broad bean shoots and roots. On the other hand, CAT activity seems to be less inducible in shoots while in roots its activity declined under higher Tl concentrations. These results show differential responses of antioxidative enzymes to Tl in roots and shoots and suggest that, depending on the enzyme activity, Tl can become inhibitory above a given concentration or after a given period of exposure. A decrease in CAT activity under Tl toxicity suggests a possible delay in removal of H_2O_2 and toxic peroxides mediated by catalase and in turn an enhancement in the free radical mediated lipid peroxidation under Tl toxicity. The increase of non-specific POX activity was shown to be strongly correlated with metal ion concentration and related biomass production and

it is generally considered as an indication of plant ageing [6]. Pyrogallol peroxidase activity in broad bean roots was several times higher than in shoots, which may be consequence of much higher accumulation of thallium in roots. Similar distribution of POX activity was also noticed in rice seedlings exposed to lead [7].

By simultanous application of Tl and K, oxidative damage to broad bean shoots and roots was suppressed by efficient antioxidative defense, thus indicating positive effect of excess K on Tl-induced oxidative stress. However, as combined treatment induced DNA damage, it seems that exogenously added potassium shows only partial ameliorative effect regarding Tl toxicity.

Another widely used indicator of stress in general, including metal toxicity, is expression of HSP70. Thallium induced no new HSP-isoforms but caused accumulation of HSP72c isoform in roots while the intensity of all three present HSP72 isoforms markedly declined under highest Tl treatment. Gradual inhibition of constitutive HSP72 isoforms in broad bean shoots was evident at all but lowest Tl treatment. Several studies show the same pattern of changes of HSP70 in response to cadmium- and copper-induced stress [24, 57].

In conclusion, as thallium is a non-redox metal, the oxidative stress induced by thallous ions is most likely an indirect effect. Absence of direct DNA damage in broad bean nuclei at 50 mg/L of Tl-acetate speaks in favor of the hypothesis. On the other hand, antioxidative system of broad bean seedlings seems to be inducible, even at lower, environmentally encountered Tl concentrations. Thus, oxidative stress characterized by increased production of ROS could be an important mechanism of Tl toxicity, though extensive research is yet needed at the molecular and subcellular levels in order to get a deeper insight into Tl toxicity.

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REFERENCES

1. Cargnelutti D, Almeri Tabaldi L, Spanevello RM, de Oliveira Jucoski G, Battisti V, Redin M, Blanco Linares CE, Dressler VL, de Moraes Flores EM, Teixeira Nicoloso F, Morsch VM, Chitolina Schetinger MR. 2006. Mercury toxicity induces oxidative stress in growing cucumber seedlings. *Chemosphere* 65:999-1006.
2. Hou W, Chen X, Song G, Wang Q, Chang CC. 2007. Effects of copper and cadmium on heavy metal polluted waterbody restoration by duckweed (*Lemna minor*). *Plant Physiol Boichem* 45:62-69.
3. Schützendübel A, Polle A. 2002. Plant responses to abiotic stresses: Heavy metal-induced oxidative stress and protection by mycorrhization. *J Exp Bot* 53:1351-1365.
4. Boscolo PRS, Menossi M, Jorge RA. 2003. Aluminium-induced oxidative stress in maize. *Phytochemistry* 62:181-189.
5. Dixit V, Pandey V, Shyam R. 2001. Differential antioxidative responses to cadmium in roots and leaves of pea (*Pisum sativum* L. cv. Azad). *J Exp Bot* 52:1101-1109.
6. Van Assche F, Clijsters H. 1990. Effects of metals on enzyme activity in plants. *Plant Cell Environ* 13:195-206.
7. Verma S, Dubey RS. 2003. Lead toxicity induces lipid peroxidation and alters the activities of antioxidant enzymes in growing rice plants. *Plant Sci* 164:645-655.
8. Cuypers A, Vangronsveld J, Clijsters H. 2001. The redox status of plant cells (AsA and GSH) is sensitive to zinc imposed oxidative stress in roots and primary leaves of *Phaseolus vulgaris*. *Plant Physiol Biochem* 39:657-664.
9. Galván-Arzate S, Santamaría A. 1998. Thallium toxicity. *Toxicol Lett* 99:1-13.

10. Heim M., Wappelhorst O, Markert B. 2002. Thallium in terrestrial environments – occurrence and effects. *Ecotoxicology* 11:369-377.
11. John Peter AL, Viraraghavan T. 2005. Thallium: A review of public health and environmental concerns. *Environ Int* 31:493-501.
12. Dmowski K, Kozakiewicz A, Kozakiewicz M. 1998. Small mammal populations and community under conditions of extremely high thallium contamination in the environment. *Ecotoxicol Environ Saf* 41:2-7.
13. Wierzbicka M, Szarek-Łukaszewska G, Grodzińska K. 2004. Highly toxic thallium in plants from the vicinity of Olkusz (Poland). *Ecotoxicol Environ Saf* 59:84-88.
14. Xiao T, Guha J, Boyle D, Liu C-Q, Zheng B, Wilson GC, Rouleau A, Chen J. 2004. Naturally occuring thallium: a hidden geoenvironmental health hazard? *Environ Int* 30: 501-507.
15. Villaverde MS, Verstraeten SV. 2003. Effects of thallium(I) and thallium(III) on liposome membrane physical properties. *Arch Biochem Biophys* 417:235-243.
16. Galván-Arzate S, Pedraza-Chaverrí J, Medina-Campos ON, Maldonado PD, Vázquez-Román B, Ríos C, Santamaría A. 2005. Delayed effects of thallium in the rat brain: Regional changes in lipid peroxidation and behavioral markers, but moderate alterations in antioxidants, after a single administration. *Food Chem Toxicol* 43:1037-1045.
17. Kwan KHM, Smith S. 1991. Some aspects of the kinetics of cadmium and thallium uptake by fronds of *Lemna minor* L. *New Phytol* 117:91-102.
18. LaCoste C, Robinson B, Brooks R. 2001. Uptake of thallium by vegetables: Its significance for human health, phytoremediation, and phytominig. *J Plant Nutr* 24:1205-1215.

19. Pötsch U, Austenfeld FA. 1985. Phytotoxicity of thallium (Tl) in culture solution part 1: Effects of Tl(I) on the growth and heavy metal contents of pea and field bean plants. *Z Pflanzenernähr Bodenkd* 148:73-82.

20. Tremel A, Masson P, Garraud H, Donnard OFX, Baize D, Mench M. 1997. Thallium in French agrosystems-II. Concentration of thallium in field-grown rape and some other plant species. *Environ Pollut* 97:161-168.

21. Lan C-H, Lin T-S. 2005. Acute toxicity of trivalent thallium compounds to *Daphnia magna*. *Ecotoxicol Environ Saf* 61:432-435.

22. Scheckel KG, Lombi E, Rock SA, McLaughlin MJ. 2004. In vivo synchrotron study of thallium speciation and compartmentation in *Iberis intermedia*. *Environ Sci Technol* 38:5095-5100.

23. Mulkey JP, Oehme FW. 1993. A review of thallium toxicity. *Vet Hum Toxicol* 35:445-453.

24. Ireland EH, Harding SJ, Bonwick GA, Jones M, Smith CJ, Williams JH. 2004. Evaluation of heat shock protein 70 as a biomarker of environmental stress in *Fucus serratus* and *Lemna minor*. *Biomarkers* 9:139-155.

25. Neumann D, Lichtenberger O, Günther D, Tschiersch K, Nover L. 1994. Heat-shock proteins induce heavy-metal tolerance in higher plants. *Planta* 194:360-367.

26. Matysik J, Alia, Bhalu B, Mohanty P. 2002. Molecular mechanisms of quenching of reactive oxygen species by proline under stress in plants. *Curr Sci* 82:525-532.

27. Günther K, Umland F. 1989. Bonding states of thallium and cadmium in thallium-treated and native rape. *J Inorg Biochem* 36:63-74.

28. Bernier SP, Silo-Suh L, Woods DE, Ohman DE, Sokol PA. 2003. Comparative analysis of plant and animal models for characterization of *Burkholderia cepacia* virulence. *Infect Immun* 71:5306-5313.

29. Fiskesjö G. 1988. The *Allium* test—an alternative in environmental studies: The relative toxicity of metal ions. *Mutat Res* 197:243-260.

30. Iriti M, Faoro F. 2008. Oxidative stress, the paradigm of ozone toxicity in plants and animals. *Water Air Soil Pollut* 187:285-301.

31. Bates LS, Waldren RP, Teare ID. 1973. Rapid determination of free proline for water stress studies. *Plant Soil* 39:205-207.

32. Gichner T, Patková Z, Száková J, Demnerová K. 2004. Cadmium induces DNA damage in tobacco roots, but no DNA damage, somatic mutations or homologous recombination in tobacco leaves. *Mutat Res* 559:49-57.

33. Heath RL, Packer L. 1968. Photoperoxidation in isolated chloroplasts. I-kinetics and stoichiometry of fatty acid peroxidation. *Arch Biochem Biophys*125:189-198.

34. Mukherjee SP, Choudhouri MA. 1983. Implications of water stress-induced changes in the levels of endogenous ascorbic acid and hydrogen peroxide in *Vigna* seedlings. *Physiol Plant* 58:166-170.

35. Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Ahn BW, Shaltiel S, Stadtman ER. 1990. Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol* 186:464-478.

36. Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248-254.

37. Beauchamp C, Fridovich I. 1971. Superoxide dismutase: Improved assay and an assay applicable to PAGE. *Anal Biochem* 44:276-287.

38. Nakano Y, Asada K. 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant Cell Physiol* 22:867-880.

39. Chance B, Maehly AC. 1955. Assay of catalases and peroxidises. In Colowick SP, Kaplan NO, eds, *Methods in Enzymology*. Academic, New York, NY, pp 764-775.

40. Aebi H. 1984. Catalase in vitro. *Methods Enzymol* 105:121-126.

41. Al-Attar AF, Martin MH, Nickless G. 1988. Uptake and toxicity of cadmium, mercury and thallium to *Lolium perenne* seedlings. *Chemosphere* 17:1219-1225.

42. Trivedi S, Erdei L. 1992. Effects of cadmium and lead on the accumulation of Ca^{2+} and K^+ and on the influx and translocation of K^+ in wheat of low and high K^+ status. *Physiol Plant* 84:94-100.

43. Siegel BZ, Siegel SM. 1976. Effects of potassium on thallium toxicity in cucumber seedlings: Further evidence for potassium-thallium ion antagonism. *Bioinorg Chem* 6:341-345.

44. Perfus-Barbeoch L, Leonhardt N, Vavasseur A, Forestier C. 2002. Heavy metal toxicity: Cadmium permeates through calcium channels and disturbs the plant water status. *Plant J* 32:539-548.

45. Saradhi A, Saradhi PP. 1991. Proline accumulation under heavy metal stress. *J Plant Physiol* 138:554-558.

46. Bassi R, Sharma SS. 1993. Proline accumulation in wheat seedlings exposed to zinc and copper. *Phytochemistry* 33:1339-1342.

47. Aspinall D, Paleg LG. 1981. Proline accumulation: Physiological aspects. In Paleg LG, Aspinall D, eds, *The Physiology and Biochemistry of Drought Resistance in Plants*. Academic, Sidney, Australia, pp 205-240.

48. Sharma SS, Dietz K-J. 2006. The significance of aminoacids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. *J Exp Bot* 57:711-726.

49. Sharma SS, Schat H, Vooijs R. 1998. In vitro alleviation of heavy metal-induced enzyme inhibition by proline. *Phytochemistry* 49:1531-1535.

50. Cordova Rosa EV, Valgas C, Souza-Sierra MM, Corrêa AXR, Radetski CM. 2003. Biomass growth, micronucleus induction, and antioxidant stress enzyme responses in *Vicia faba* exposed to cadmium in solution. *Environ Toxicol Chem* 22:645-649.

51. Ünyayar S, Çelik A, Çekiç FÖ, Gözel A. 2006. Cadmium-induced genotoxicity, cytotoxicity and lipid peroxidation in *Allium sativum* and *Vicia faba*. *Mutagenesis* 21:77-81.

52. Aoyama H, Yoshida M, Yamamura,Y. 1988. Induction of lipid peroxidation in tissues of thallous malonate-treated hamster. *Toxicology* 53:11-18.

53. Hanzel CH, Verstraeten SV. 2006. Thallium induces hydrogen peroxide generation by impairing mitochondrial function. *Toxicol Appl Pharmacol* 216:485-492.

54. Korotkov SM, Lapin AV. 2003. Thallium induces opening of the mitochondrial permeability transition pore in the inner membranes of rat liver mitochondria *Doklady Biochemistry and Biophysics* 392:247-252.

55. Blokhina O, Virolainen E, Fagerstedt KV. 2003. Antioxidants, oxidative damage and oxygen deprivation stress: A review. *Ann Bot* 91:179-194.

56. Reinheckel T, Noack H, Lorenz S, Wiswedel I, Augustin W. 1998. Comparison of protein oxidation and aldehyde formation during oxidative stress in isolated mitochondria. *Free Radical Research* 29:297-305.

57. Bačkor M, Gibalová A, Budová J, Mikeš J, Solár P. 2006. Cadmium-induced stimulation of stress-protein hsp70 in lichen photobiont *Trebouxia erici*. *Plant Growth Regul* 50:159-164.

Figure Legends

Figure 1. Proline content in *Vicia faba* shoots (□) and roots (■) under control (C1) and stress – 0.5 mg/L (1), 1 mg/L (2), 5 mg/L (3), 10 mg/L (4) of thallium-acetate, combined treatment (5 mg/L thallium-acetate and 10 mg/L potassium-acetate) (5) and 10 mg/L potassium-acetate (C2) – conditions after 72-h growth period. Values are mean ± standard deviation based on six replicates. Bars with different letters (lower case letters for shoots and upper case letters for roots) are significantly different at $p < 0.05$.

Figure 2. Hydrogen peroxide (**A**), malondialdehyde (**B**) and reactive carbonyl (C=O) groups (**C**) contents in *Vicia faba* shoots (□) and roots (■) under control and stress conditions after 72-h growth period. Values are mean ± standard deviation based on six replicates. Bars with different letters are significantly different at $p < 0.05$. For abbreviations explanation of control and stress conditions refer to Figure 1.

Figure 3. Superoxide dismutase (**A**), ascorbate peroxidase (**B**), pyrogallol peroxidase (**C**) and catalase (**D)** activities in *Vicia faba* shoots (□) and roots (■) under control and stress conditions after 72-h growth period. Values are mean ± standard deviation based on six replicates. Bars with different letters are significantly different at $p < 0.05$. For explanation of control and stress conditions refer to Figure 1.

Figure 4. Patterns of broad bean shoot and root heat-shock protein of 70 kDa (HSP70) control (C1) and stress – 0.5 mg/L (1), 1 mg/L (2), 5 mg/L (3), 10 mg/L (4) of thallium-acetate, combined treatment (5 mg/L thallium-acetate and 10 mg/L potassium-acetate) (5) and 10 mg/L potassium-acetate (C2) – conditions after 72-h growth period.

Table 1. Dry weight (mg), relative water status, thallium (Tl) and potassium (K) contents (mg/kg dry wt) of *Vicia faba* roots and shoots.

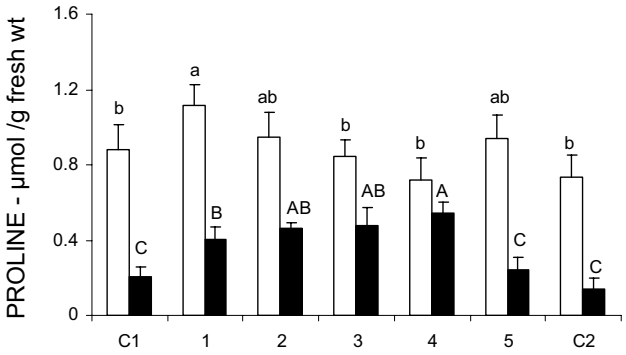
Concn. (mg/L)	Shoot				Root			
	Dry wt	Relative water status	Thallium content	Potassium content	Dry wt	Relative water status	Thallium content	Potassium content
Control	40.51 BC	0.898 BC	0.37 D	19750 A	25.40 D	0.936 A	0.51 A	12804 B
Tl 0.5	26.96 D	0.933 A	1.49 CD	17707 ABC	27.77 CD	0.930 A	73.53 B	11450 BC
Tl 1	36.78 C	0.908 B	3.71 BC	15931 BC	28.68 BC	0.928 AB	351.88 C	10402 C
Tl 5	39.89 BC	0.900 BC	6.40 B	16113 BC	29.43 AB	0.926 BC	1145.70 D	9426 CD
Tl 10	51.57 A	0.871 D	51.28 A	15307 C	32.02 A	0.919 C	2315 E	8834 D
Tl 5 + K 10	44.42 B	0.889 C	4.54 B	18757 AB	31.03 AB	0.922 BC	1212.30 D	14880 A
K 10	40.42 BC	0.898 BC	0.18 D	20287 A	26.92 CD	0.933 A	0.43 A	13263 AB

Numbers are means of three replicates. Values followed by the same letter are not significantly different ($p < 0.05$).

Table 2. Effect of thallium (Tl) and potassium (K) acetate on average median tail moment values ± S.E. of cellular and acellular Comet assay.

Concn. (mg/L)	Cellular		Acellular
	Shoot	Root	
	Tail moment (µm)	Tail moment (µm)	Tail moment (µm)
Tl 0	1.97 ± 0.293 B	1.28 ± 0.347 D	1.49 ± 0.222
Tl 0.5	2.75 ± 0.319 B	9.42 ± 0.599 C	1.03 ± 0.122
Tl 1	3.80 ± 0.264 B	14.37 ± 0.856 C	1.71 ± 0.310
Tl 5	5.01 ± 0.556 B	13.10 ± 0.668 B	1.69 ± 0.240
Tl 10	18.78 ± 0.123 A	45.39 ± 0.708 A	1.48 ± 0.153
Tl 5 + K 10	3.49 ± 0.186 B	19.73 ± 0.670 B	1.47 ± 0.204
Tl 50			1.72 ± 0.245
K 10	1.62 ± 0.297 B	1.32 ± 0.531 D	1.28 ± 0.244

Values followed by the same letter are not significantly different ($p < 0.05$).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1.2
0.8
0.4
0
b
C
a
B
ab
AB
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b
A
ab
C
b
C
C1
1
2
3
4
5
C2

Figure 1.

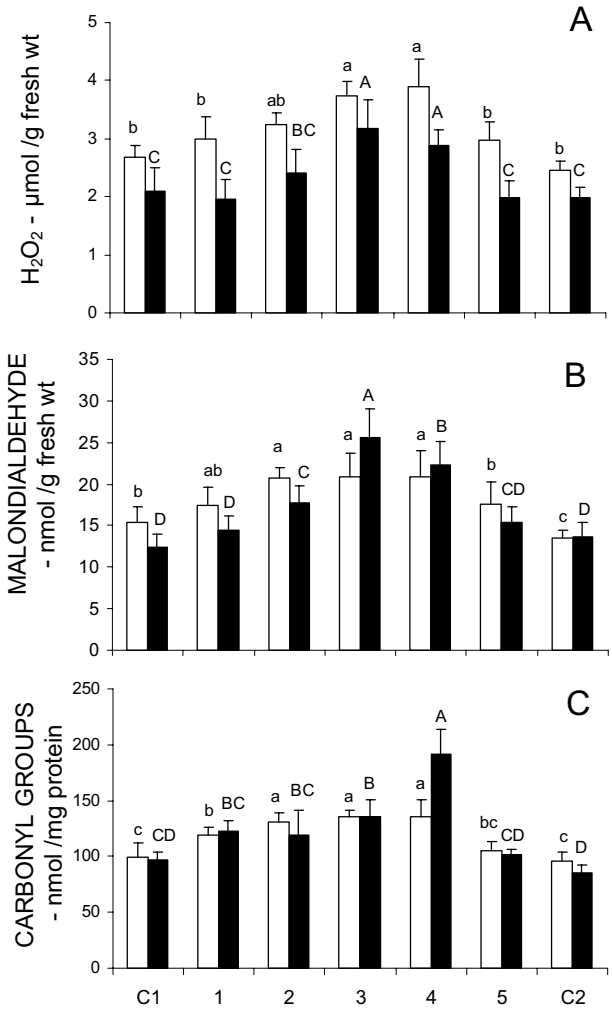
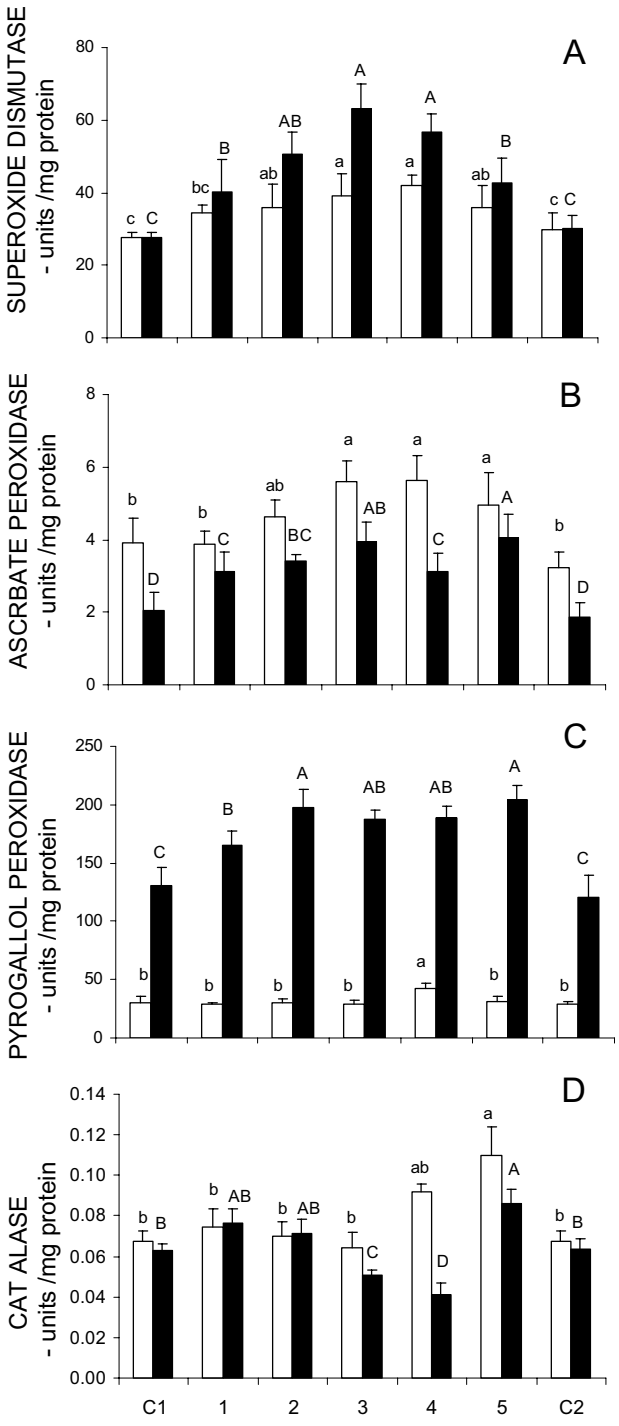

A
B
C
H_2O_2 - µmol /g fresh wt
MALONDIALDEHYDE - nmol /g fresh wt
CARBONYL GROUPS - nmol /mg protein
C1
1
2
3
4
5
C2

Figure 2.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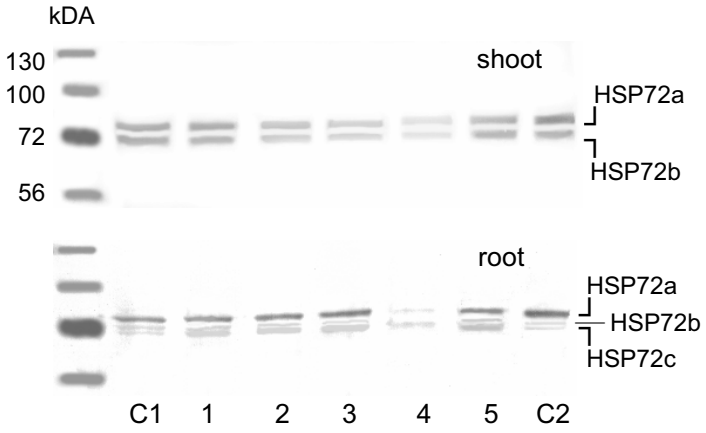
- units /mg protein
A
ASCRBATE PEROXIDASE
- units /mg protein
B
PYROGALLOL PEROXIDASE
- units /mg protein
C
CAT ALASE
- units /mg protein
D
C1
1
2
3
4
5
C2

Figure 3.


kDA
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100
72
56
shoot
HSP72a
HSP72b
root
HSP72a
HSP72b
HSP72c
C1
1
2
3
4
5
C2

Figure 4.