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Localization and Study of Histochemical Effects of Excess Mn in Sunflower (*Helianthus annuus* L. cv. Azarghol) Plants

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Abstract

A high capacity for accumulation of Mn was reported for sunflower plants. Localization of excess Mn is therefore of special interest for understanding metal tolerance mechanisms in this species. In this study, structural and histochemical alterations caused by Mn accumulation in leaves were investigated in sunflower (*Helianthus annuus* L. cv. Azar-ghol) plants grown in nutrient solution. In the presence of excess Mn (up to 100 μM) shoot and root accumulated up to 4-5 mg Mn g^{-1}DW concomitant with an increased activity of peroxidase, but not catalase. Symptoms were observed in mature leaves of plants as small dark-brown to black spots associated with the leaf trichomes. In the short uni- or multi-cellular leaf hairs the entire trichome was blackened, while there was a blackened basal cell or tip cell in the long linear multi-cellular trichomes. No dark deposition was observed either in the tip or in the base of the spiral multi-cellular trichomes. Epi-illumination method revealed a dense dark discoloration of small veins of areoles in mature leaves of Mn treated plants with a high auto-fluorescence, suggesting accumulation of phenolics. Histochemical methods for callose, lipids, lignin and proteins showed no change in Mn treated leaves; while a significant decrease of starch grains was observed using polysaccaride identification test. Bleaching of dark spots by ethanol and lack of auto-fluorescence in the locations of leaf trichomes as well as result of histochemical methods for free and bound phenolics suggested strongly that the dark spots are the locations of MnO_2 deposits.

Keywords: Mn toxicity; Oxidized Mn; Phenolic compounds; Sunflower

Introduction

Manganese (Mn) is an essential micronutrient for plants; however, excess amounts of this heavy metal induce a wide range of biochemical and physiological processes and alter photosynthesis, protein metabolism and membrane integrity [32]. Although Mn is not a common pollutant in soils, various soil conditions often

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present in acid and volcanic soils or submergence can lead to Mn reduction and create Mn toxicity in many natural and agricultural systems [19].

Plant species differ considerably in their susceptibility to Mn toxicity and the critical toxicity concentration (associated with 90% maximum yield) varies greatly among plant species. In a solution culture study on 13 crop and pasture species, a wide range of critical toxicity concentration from 200 (maize) to 5300 (Sunflower) mg Kg^{-1} dry mass have been reported [15].

Three possible mechanisms have been suggested for the tolerance of plants to high levels of available Mn in the root environment (i) low levels of Mn uptake by the roots, (ii) retention of Mn in the roots, and (iii) ability of the tops to tolerate high tissue Mn concentrations. Result of works on sunflower plants suggested that an extraordinary high tolerance of this crop species to excess Mn resulted from an ability to tolerate high concentrations of Mn in the shoot rather than preventing excess Mn from entering the tops [19].

A high tolerance to Mn toxicity in combination with higher Mn accumulation in leaves is characteristic for Mn hyperaccumulator species growing naturally on Mn rich soils. To date, 12 species mainly woody shrubs and trees from subtropical areas have been designated as Mn hyperaccumulators [8]. Compared with other crop species, higher tolerance and capacity for accumulation of Mn was reported for sunflower plants [15].

The extraordinary accumulation of metals in hyperaccumulators apparently involves compartmentation of toxic metals at the cellular and sub-cellular levels [29]. Metal complexes must be stored where they cannot readily dissociate and where they will not interrupt normal metabolic activities of the cell. Localization studies are therefore an essential part of understanding the mechanisms involved in metal tolerance and metal hyperaccumulation in these plants. Such studies provide the opportunity to identify sites of detoxification and storage of metal complexes [5]. To date, localization studies of hyperaccumulators have shown that shoot epidermal tissue and surface structures such as trichomes are the most important sites of storage of metals [29, 48].

Manganese toxicity symptoms in plants appear first in shoots and are often more sensitive toxicity parameters than vegetative growth [32]. For many species such as barley [46], bush bean [27], sunflower [6] and cow pea [25] first Mn toxicity symptoms are dark brown speckles on old leaves. In later stages Mn toxicity may also lead to leaf yellowing, desiccation and shedding of old leaves [26].

Mn is compartmentalized in either the cell walls of the epidermis, collenchyma, bundle sheat cell, and/or vacuoles and, as such, it is isolated from metabolically active compartments such as the cytosol, mitochondria and chloroplast [35].

It was suggested that the oxidation of excess Mn in plant shoots may be alternative mechanism for plant Mn tolerance. Oxidized Mn is probably metabolically inactive since plants contained a relatively high Mn concentration in the tissues [6]. Peroxidases involve probably in the oxidation of Mn (II) to Mn (IV) and its deposition in the cells [17]. Experimental evidences show that, in bean [27] and cucumber [24] the brown Mn toxicity speckles contain oxidized Mn, but in cowpea these brown spots are locations of oxidized phenolic compounds [44].

Uptake and accumulation of heavy metals at higher concentrations, not only result in toxicity symptoms and affect metabolic processes, but also cause structural and ultrastructural changes. For heavy metals other than Mn, reduction in mesophyll cell size by Zn [48], break down of chloroplasts by Cd [2], reduction of area of leaf blades by Pb [43] toxicity were reported.

Because of the effect on plant metabolism, heavy metal toxicity may affect some histochemical properties of metal accumulating tissues. Callose formation, reduction in protein, lipids, starch or pigments [23] in the cells accumulating high amounts of heavy metals are expected. As another Mn toxicity symptom, callose deposition has been identified around the brown speckles in cow pea [45].

Information is rare on the influence of heavy metals in general and Mn in particular on structure and histochemistry of accumulating leaves. Despite of the numerous investigations carried out on the physiology and biochemistry of Mn accumulation in plants [23], precise information on the histochemical effect of Mn is limited.

In the present study, morphologic and structural alterations promoted by Mn accumulation in leaves were investigated in nutrient solution grown sunflower plants. This species is well known for a high Mn toxicity tolerance compared with other crop plants [32]. Mn toxicity symptoms are further characterized histochemically.

Materials and Methods

Plants Culture

Seeds of sunflower (*Helianthus annuus* L. cv. Azarghol) provided by Seed and Plant Improvement Institute (SPII) (Karaj, Iran), were surface-sterilized and germinated in the dark on sand moistened with distilled water and 0.05 mM $CaSO_4$. The 7-day-old seedlings

with uniform size were transferred to hydroponic culture [22] in plastic container with 2 L of nutrient solution (50%) and pre-cultured for 3 days. Manganese treatments were started in 10-day-old plants. Seedlings were treated with four levels of $MnSO_4$, 0 (control) 50, 100 and 300 µM. Growth of plants, chlorophyll concentration and activity of enzymes were determined in plants grown in the presence of excess Mn up to 100 µM. Microscopic studies were carried out in plants treated with 100 or 300 µM Mn in parallel with control ones. Nutrient solutions were completely changed every 3 days, pH of the medium was adjusted at 6.5 and controlled every day.

Experiment was conducted using complete randomized block design with four replications in a growth chamber with a temperature regime of 25°/18°C day/night, 14/10 h light/dark period and relative humidity of 70/80% under a photon flux density of 300 µmol m^{-2} s^{-1} that was measured by a quantum sensor attached to the leaf chamber of a gas exchange unit (LCA-4, ADC Bioscientific Ltd., UK).

Harvest and Determination of Mn in Plant Organs

Two weeks after treatment, plants were harvested. For removing of the apoplasmic Mn from roots, plants were placed for 1h in 5 mM $CaCl_2$+25% nutrient solution. For determination of Mn content, oven-dried samples were ashed in a muffle furnace at 550°C for 8h, resolved in HCl and made up to volume by distilled water. Metal concentration was determined by atomic absorption spectrophotometry (Shimadzu, AA 6500). Chlorophyll concentration was measured spectrophotometrically in the third pair of leaves after a 48 h extraction in N,N-dimethylformamide [37]. The absorbance of chlorophyll was measured at 664, 647 and 603 nm and chlorophyll concentration was calculated using following formula: Total chlorophyll (µg ml^{-1}) = 8.24 A_{644}+23.97 A_{647}–16.64 A_{603}.

Determination of Peroxidase and Catalase Activity

Fresh leaf samples were used for enzyme extraction and measurement of protein concentration. Samples were ground in extraction buffer using pre-chilled mortar and pestle. Each enzyme assay was tested for linearity between the volume of crude extract and the measured activity.

Peroxidase

Peroxidase (POD, EC 1.11.1.7) activity was determined using the guaiacol test [9]. The tetraguaiacol formed in the reaction has an absorption maximum at 470 mm, and thus the reaction can be readily followed photometrically. The enzyme was extracted by 10 mM phosphate buffer (pH=7.0), and assayed in a solution contained 10 mM phosphate buffer, 5 mM H_2O_2 and 4 mM guaiacol. The reaction was started by addition of the enzyme extract at 25°C and was followed 2 min after starting the reaction. The enzyme unit was calculated as enzyme protein required for the formation of 1 µM tetraguaiacol for 1 min.

Catalase

Catalase (CAT, EC 1.11.1.6) activity was assayed spectrophotometrically by monitoring the decrease in absorbance of H_2O_2 at 240 nm [31]. The enzyme was extracted in 50 mM phosphate buffer (pH=7.0). The assay solution contained 50 mM phosphate buffer and 10 mM H_2O_2. The reaction was started by addition of enzyme aliquot to the reaction mixture and the change in absorbance was followed 2 min after starting the reaction. Unit activity was taken as the amount of enzyme, which decomposes 1 M of H_2O_2 in one min.

Total Protein Concentration

Soluble proteins were determined as described by Bradford [7] using a commercial reagent (Sigma) and BSA (Merck) as standard.

Statistical analyses were carried to determine significant differences between treatments using Tukey test (p<0.05).

Morphological and Structural Studies

Preliminary observations were made for morphological characterizing of leaf symptoms. Both fresh and cleared leaves [3] were used. Leaf symptoms were examined with naked eye or using dissection microscopy.

For structural studies, leaf samples of 5-10 mm around midrib were excised from the middle portion of the third to fifth old leaves (having severe visual symptoms) and were immediately fixed. Three different fixatives were used: formalin-acetic acid-alcohol (FAA) [20], Regaud [39] and formal-calcium [20]. Samples then were dehydrated and infiltrated using alcohol and xylol series respectively and were paraffin embedded [20].

Sections of 20 to 40 µm diameter were made using a R. Jung Heidelberg microtome and then counter-stained with Safranin-Fast green [3].

Histochemical Studies

Leaf sections were stained with proper stains for

visualizing different compounds by light or fluorescence microscopy. The stains used were as follow [20]: Periodic acid-Schiff (PAS) reaction for localization of polysaccharides, Coomassie-brilliant blue R250 for proteins, Nile blue sulfate for lipids, Anillin-blue for callose detection by light and fluorescent microscopy, potassium iodide for starch, Phloroglucin for lignin, nitrous acid, ferric chloride test and modified Feder and OBerien's indicator [18] for phenolics.

Structural and histochemical studies were undertaken using light microscopy with Nikon E1000 or SMS 1500 equipped with digital still camera. Fluorescence microscopic studies were performed using an Olympus BX51 microscope equipped with BX-RFA fluorescence illuminator (Olympus Optical Co., Japan).

Results

Plant growth was affected significantly by Mn treatment depending on its concentration. Low level of Mn in the medium (50 µM) did not result in reduction of plants biomass, shoot and root dry weight rather increased up to 34% and 16% respectively. Chlorophyll concentration of leaves was also increased significantly by Mn treatment of 50 µM. Higher concentrations of Mn, in contrast, inhibited shoot and root growth by about 6% and 31% respectively, which was significant for root (Table 1).

Shoot and root accumulated great amounts of Mn, which reached up to 4-5 mg g^{-1} DW. Even for plants with growth stimulation, concentration of Mn ranged from 1.8 to 3.6 mg g^{-1} DW for roots and leaves respectively (Table 2).

Protein concentration was slightly higher in plants under Mn toxicity. Specific activity of peroxidase and catalase was affected by toxic concentration of Mn differently. Activity of peroxidase increased but that of catalase decreased significantly in the presence of toxic concentration of Mn (Table 3).

Plants treated with Mn concentration of 50 µM did not show any symptom of Mn toxicity. Symptoms were observed in plants treated with 100 µM and higher (300 µM). With high concentrations of Mn in solution, small dark-brown to black spots (<0.5 mm in diameter) developed on the lower stem and on the petioles and blades of the lower (fully expanded mature) leaves. The small dark spots which were readily seen with the naked eye were not necrotic, and were clearly associated with trichomes (Fig. 1).

In younger leaves, in contrast, dark brown lesions (>5 mm) were developed. These symptoms appeared to be unrelated to the small dark colored spots associated with the trichomes in mature leaves. On the upper leaves of plants a marked yellow veinal cholosis developed accompanied by leaf crinkling. Subsequently, substantial areas of interveinal cholosis developed on the upper leaves followed by the development of large irregular patches of light brown-colored necrotic tissue (Fig. 2).

Leaves possess numerous trichomes with higher density on and around midrib. Three major groups of leaf trichomes were identified including simple short uni- or multi-cellular trichomes, long multi-cellular trichomes and spiral multi-cellular trichomes. Multi-cellular trichomes are abundant on the upper (adaxaial) epidermis (Fig. 3).

Table 1. Dry weight (mg $plant^{-1}$) of shoot and root and leaf chlorophyll concentration (mg g^{-1} FW) of sunflower (*Helianthus annuus* L. cv. Azarghol) grown in the presence of toxic concentrations of Mn in hydroponic medium. The means refer to 4 repetitions ± SD. Data of each column followed by the same letter are not significantly different (p<0.05)

Mn Treatment (µM)	Shoot DW	Root DW	Chlorophyll
Control	1714±199 ^{b}	375±126 ^{ab}	1.11±0.09 ^{b}
50	2300±104 ^{a}	437±18 ^{a}	1.27±0.06 ^{a}
100	1606±292 ^{b}	258±4 ^{b}	1.21±0.05 ^{ab}

Table 2. Mn concentration (µg $plant^{-1}$) in shoot and roots of sunflower (*Helianthus annuus* L. cv. Azarghol) grown in the presence of toxic concentrations of Mn in hydroponic medium. The means refer to 4 repetitions ± SD. Data of each column followed by the same letter are not significantly different (p<0.05).

Mn Treatment (µM)	Shoot Mn	Root Mn
Control	100±15 ^{c}	46±21 ^{c}
50	3569±25 ^{b}	1856±12 ^{b}
100	4758±13 ^{a}	4526±31 ^{a}

Table 3. Protein concentration (mg $plant^{-1}$) and activity of peroxidase (µmol Guaiacol mg pro^{-1} min^{-1}) and catalase (µmol H_2O_2 mg pro^{-1} min^{-1}) in leaves of sunflower (*Helianthus annuus* L. cv. Azarghol) grown in the presence of toxic concentration of Mn in hydroponic medium. The means refer to 4 repetitions ± SD. Data of each column followed by the same letter are not significantly different (p<0.05).

Mn Treatment (µM)	Protein	Peroxidase	Catalase
Control	280±48 ^{a}	15.5±6.4 ^{b}	7201±52 ^{a}
100	340±97 ^{a}	27.6±9.4 ^{a}	678±98 ^{b}

Four different patterns of darkening were observed in affected leaves depending on trichome type. In the short uni- or multi-cellular leaf hairs the entire trichome was blackened, while in long multi-cellular trichomes only one or few basal cells or a tip cell was blackened. No dark deposition was observed either in the tip or in the base of the spiral trichomes (Fig. 4).

Figures obtained after application of epi-illumination method showed also different patterns of darkening depending on trichome types. Using clearing method, dark cell walls in affected trichomes and the epidermal cells around them were observed. Interestingly, brown accumulations were observed in the tips of long multi-cellualr trichomes (Fig. 5).

Epi-illumination method revealed a dense dark discoloration of small veins of areoles in mature leaves of Mn treated plants, while such dark colors were not seen either in midrib or branches. Under fluorescent microscope such dark accumulations showed a high auto-fluorescence (Fig. 6).

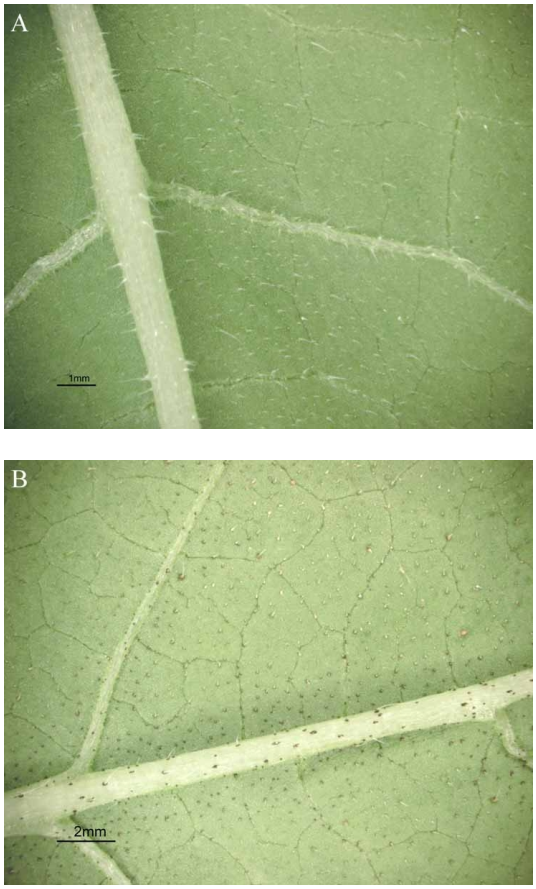

Figure 1. Surface view of mature leaves of sunflower affected by Mn toxicity showing small dark spots associated with trichomes.

Mn treatment did not alter leaf anatomy, however, intercellular spaces in Mn treated plants were significantly enlarged (Fig. 7). Histochemical methods for identification of polysaccharides (PAS method) showed low reaction in Mn treated leaves (Fig. 8). This observation was confirmed using potassium-idodide stain indicating considerable reduction in the amount of starch grains in mesophyll cells (Fig. 9). Other mentioned histochemical methods used in this work showed Mn toxicity imposed no change in protein, lipids and lignin synthesis or deposition. Furthermore, callose test and the methods for identification of free and bound phenolics indicated, Mn treatment had no effect on callose synthesis or pheolics accumulation. Thus the dark spots did not result from phenolics accumulation in affected leaves. We noted that the black spots were completely removed by fixing samples in FAA, but not by non-ethanolic ones such as Regaud and formal-calcium. This observation strongly suggested that the dark spots are the locations of oxidized Mn. Lack of auto-fluorescence in the locations of leaf trichomes (Fig. 6) confirmed also this hypothesis.

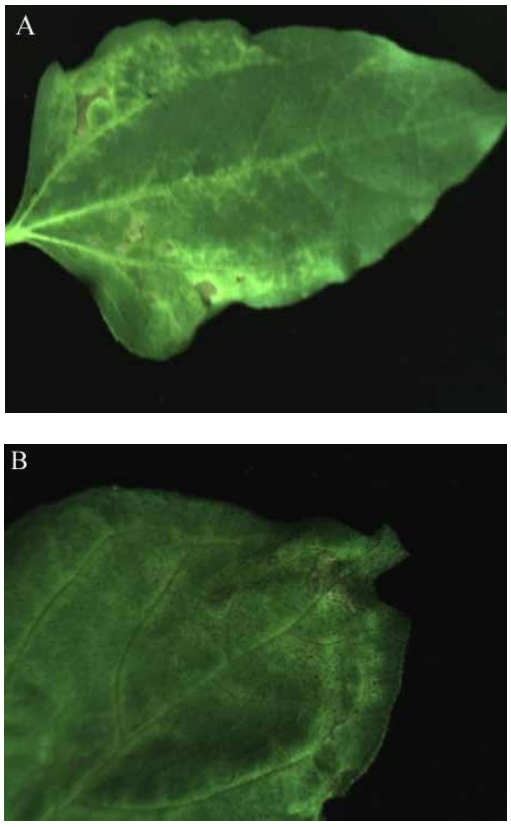

Figure 2. Surface view of young leaves of sunflower affected by Mn toxicity. Leaf cholorosis (A), leaf crinkling and light brown-colored necrotic patches (B).

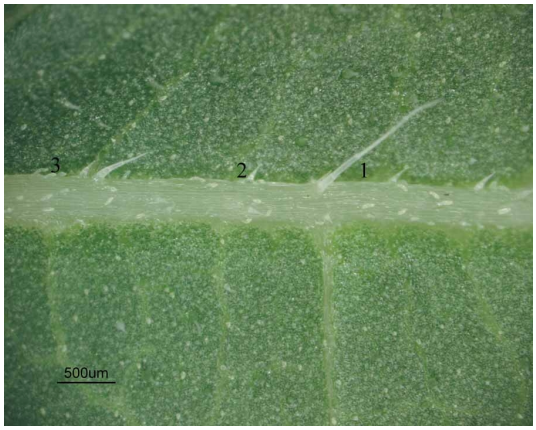

Figure 3. Adaxial surface of sunflower leaf showing three different types of trichomes. Long multi-cellular (1), short uni- or multi-cellular (2) and spiral multi-cellular trichomes (3) are detectable.

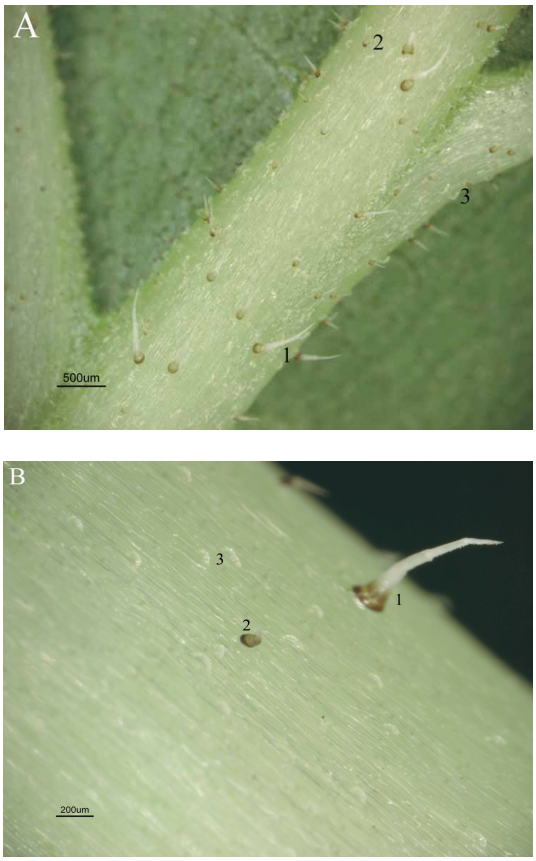

Figure 4. Different patterns of darkening in mature leaves of sunflower affected by Mn toxicity. Basal or tip blackening in long multi-cellular trichomes (1), entire blackening in short uni- and multi-cellular hairs (2) and the lack of deposition in the spiral trichomes (3).

Discussion

Plant species are greatly different in tolerance to Mn toxicity that was reflected in a wide range of their critical toxicity content [32]. In this work Mn concentration up to 50 µM in the medium caused rather a significant stimulation of shoot and root growth. Growth stimulation in the presence of higher concentrations of Mn was also demonstrated in our previous work and attributed to lower transpiration and an improved water balance [22]. In addition of growth stimulation in response to moderate levels of excess Mn, sunflower plants showed a considerable tolerance to higher concentrations of this metal. Shoot dry weight was only slightly reduced in the presence of excess Mn up to 100 µM. Species such as maize, pea and soybean were reported to be more susceptible than sunflower to higher Mn concentrations in the medium [15].

Sunflower plants accumulate considerable amounts of Mn in root and shoot. It was proposed that accumulation of heavy metals in leaves show a special ability of plants to transport absorbed metals and store them in their above-ground parts [14]. Although Mn concentration of leaves in sunflower plants treated with 100 µM Mn was lower than the standard value for hyperaccumulation (10000 $\mu g g^{-1}$DW), it was orders of magnitude higher than Mn concentration in normal plants on mineralized soils (1000 $\mu g g^{-1}$DW) [8]. It suggested that, sunflower plants have a high potential for Mn accumulation in leaves and implies involvement of mechanisms for tolerance of great amounts of Mn in leaf tissues.

Plants distribute metals internally in many different ways. They may localize heavy metals mostly in roots and stems or accumulate and store them in nontoxic form for latter distribution and use. A mechanism of tolerance or accumulation in some plants apparently involves binding potentially toxic metals at cell walls of roots and leaves away from sensitive sites within the cell or storing them in vacuolar compartment [36]. Sequestration of metal ions and metal-chelate complexes is an important aspect of metal ion detoxification in accumulator plants. Preferential accumulation in the leaf epidermis, especially in the vacuoles, has been demonstrated for Cd [42] and Zn [41, 29]. In a number of plants species there is evidence that also trichomes could play a role in detoxification as found for Cd [30], Zn [48] and Pb [33] and Cu [1]. Blamely and coworkers [6] suggested that Mn is accumulated and stored by the trichomes in sunflower leaves, possibly as insoluble higher oxides.

In our work Mn was found to accumulate in the base of leaf trichomes and some epidermal cells around

trichomes. The solubility and bleaching of spots by the ethanol containing fixatives confirmed the chemical form of Mn deposits. In addition, evidence for the localized accumulation of oxidized Mn in and around the trichomes of leaves was provided by fluorescence microscopy. Lack of auto-flourecence effect in the location of trichomes revealed that, in contrast to species such as cowpea [44] polyphenols is not the reason of dark brown color of spots in the leaves.

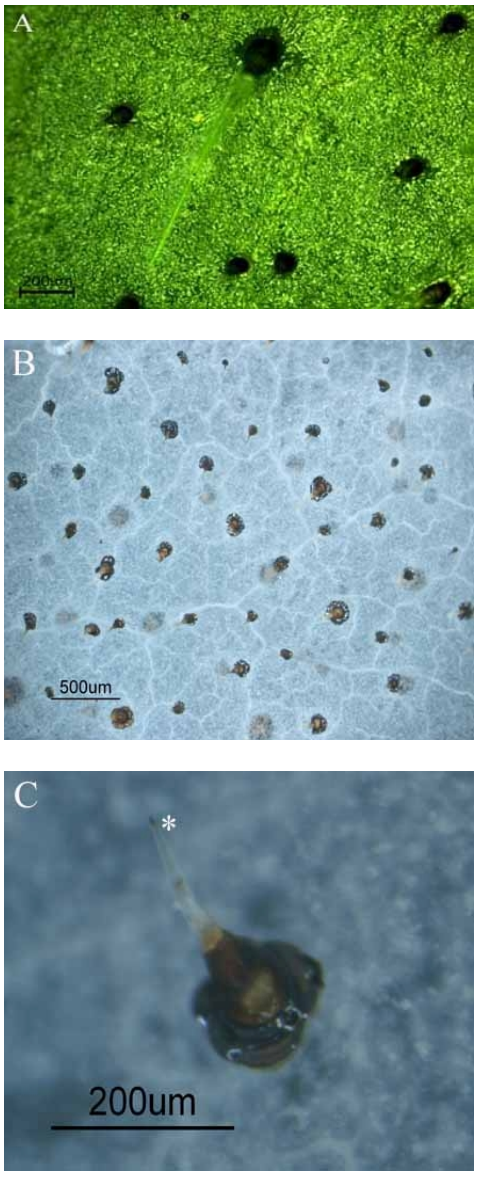

Figure 5. Surface view of mature leaf of sunflower under Mn toxicity after application of epi-illumination (A) or clearing (B and C) methods. Affected trichomes and epidermal cells around them show darkening of cell walls. Darkening of tip cells (*).

Acceleration in the enzyme activities such as peroxidase and catalase are believed to play a metabolic role under conditions of metals stress and therefore may have a subtle role in metal tolerance [38]. Peroxidases are considered to be heavy metal stress-related enzymes and are used as stress markers in metal poisoning situations [28]. It was shown that, increase in their activity protects plants to various stress factors [21]. In many plant species, excessive uptake of heavy metals such as Ni, Pb and Cd induces a strong increase of peroxidase activities and qualitative changes to their isozyme patterns [10,34]. Although the effects of heavy metals on the activity of oxygen radical detoxifying peroxidases have been widely reported, their involvement in the defense mechanisms of plant tissues against metal-induced damages remains controversial [10]. In the present work, unspecific activity of peroxidase assayed with guaiacol as a universal

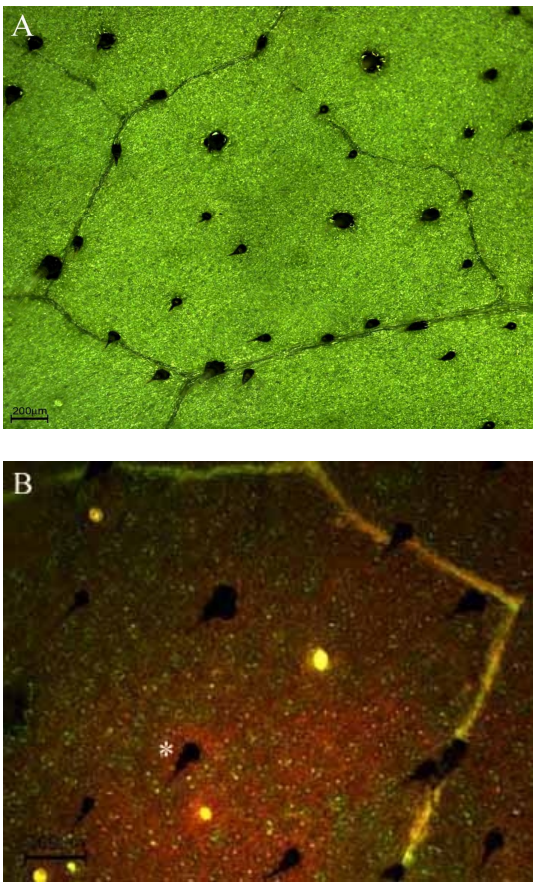

Figure 6. Fluorescent microscopy of Mn-toxicity affected sunflower leaves. Dark discoloration of small veins of areoles observed after application of epi-illumination method (A). Auto-fluorescence in small veins (B) and the lack of that in dark accumulations around trichomes (*).

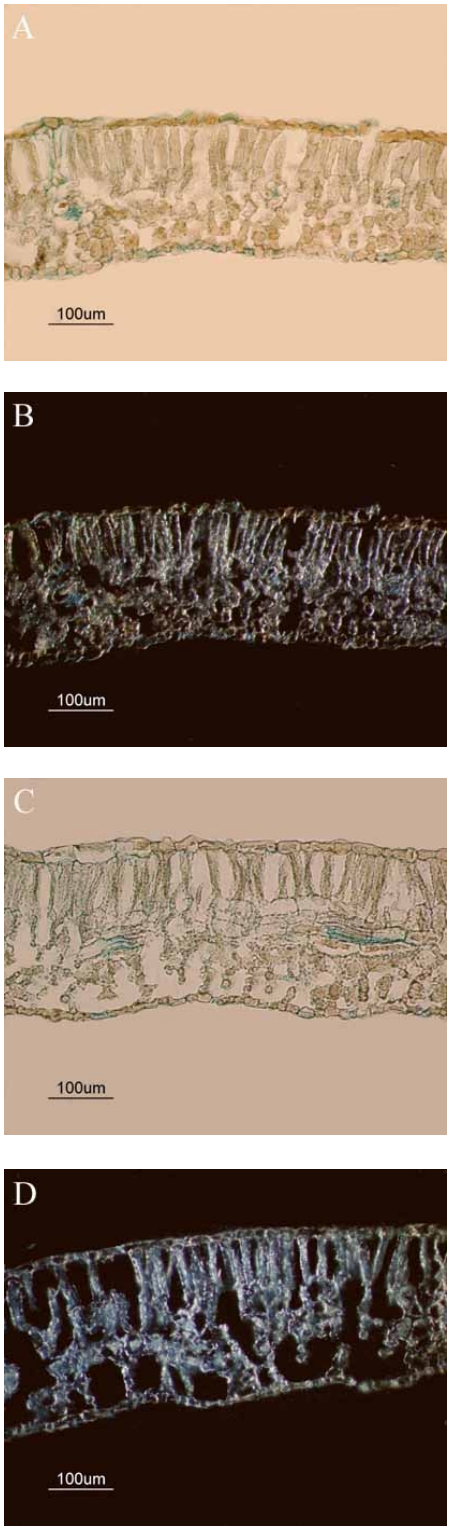

Figure 7. Cross sections of sunflower leaves under bright (A,C) and dark field (B,D) microscopy showing an enlargement of intercellular spaces in Mn treated leaves (C,D) compared with control (A,B). Regaud fixation, safranin-fast green staining.

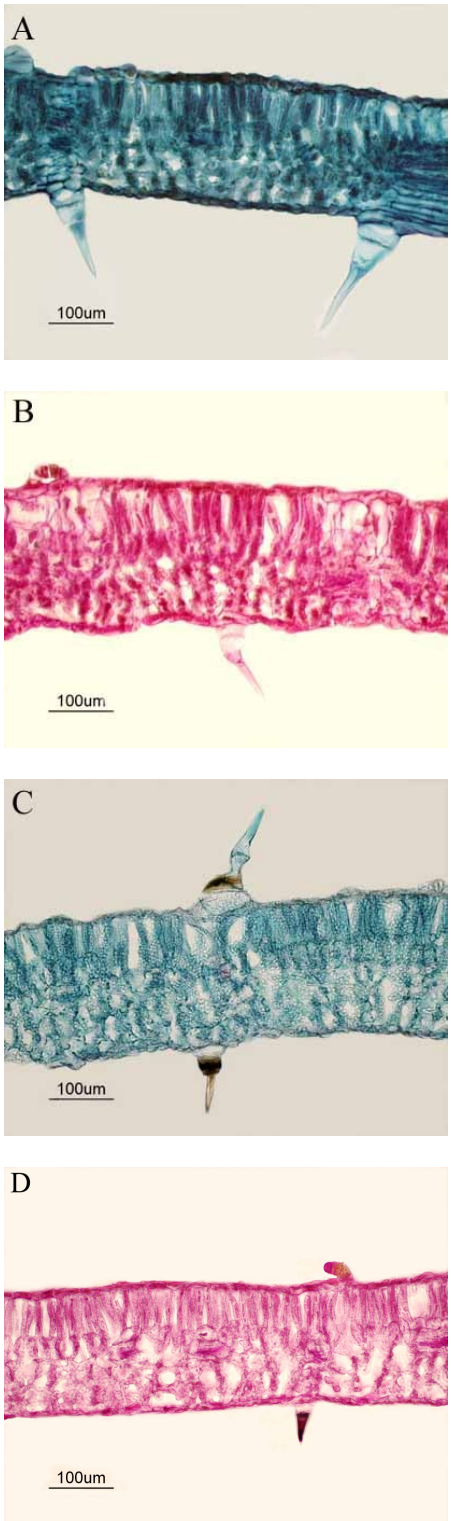

Figure 8. Cross sections of sunflower leaves. Sections from Mn-treated leaves (C,D) show lower reaction compared with control (A,B). Regaud fixation, safranin-fast green (A,C) or PAS (B,D) staining.

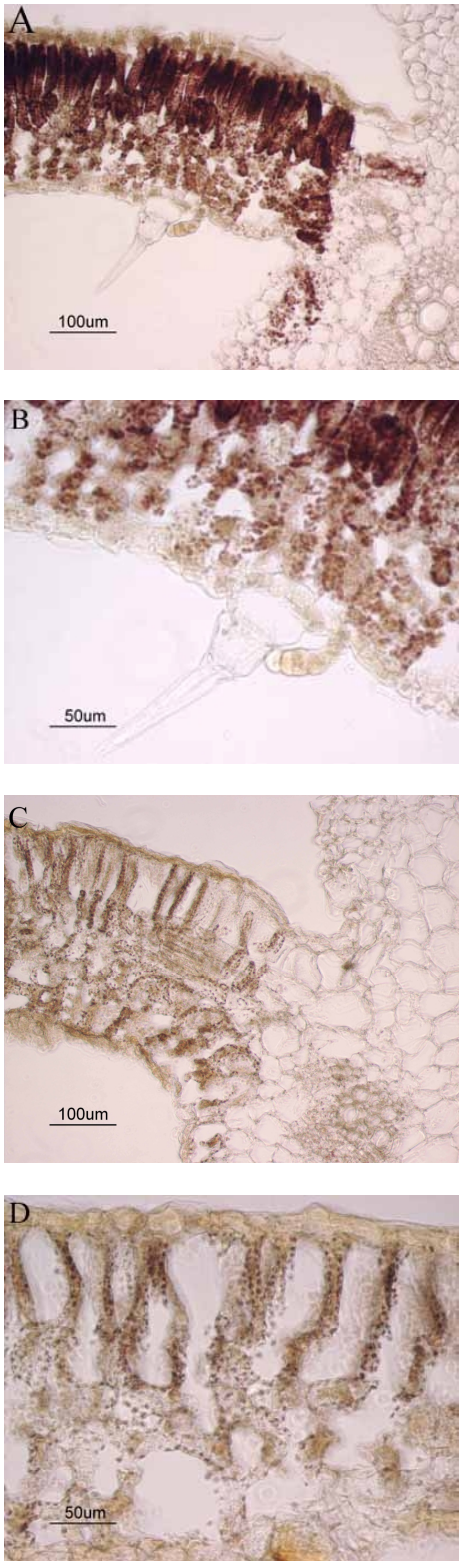

Figure 9. Cross sections of sunflower leaves. Lower accumulation of starch grains in mesophyll cells of leaves under excess Mn (C,D) compared with control (A,B). Regaud fixation, iodine-potassium iodide staining.

substrate, was stimulated by toxic concentration of Mn in the medium. Guaiacol peroxidase can exhibit activity of ascorbate peroxidase, coniferyl alcohol peroxidase, NADH oxidase and IAA oxidase. The individual activity of these enzymes could not be distinguished from the soluble pool in our extraction procedure. Cell wall bound peroxidases involve in polymerization of phenolics and formation of lignin. It was proposed that, higher activity of this enzyme accelerate lignification of tissues under stress conditions, which could be in turn the reason of lower susceptibility of some species to excess amount of heavy metals [11,12]. Some evidences were provided for involvement of peroxidases in deposition of oxidized Mn and phenolics in cowpea [17].

The starch content was significantly reduced by Mn treatment indicating an inhibition in the formation and/or accumulation of starch with uptake of Mn at higher concentrations. The starch content was compared qualitatively based on microscopic observations only. Reduction of net assimilation rate could be the reason of reduced starch accumulation in leaves. In our previous work reduction of leaf photosynthesis by excess Mn in sunflower was reported and it was demonstrated that both stomatal limitation and inhibited photochemistry of leaves are involved in inhibition of net assimilation rate in the presence of excess Mn [22]. However, in contrast to sunflower, in susceptible species such as pea dramatic structural changes was observed in chloroplasts which are associated with increased starch grains, suggesting that the transport of photoassimilates out of choroplasts may be inhibited [13].

Blamely and coworkers [6] suggested that Mn is accumulated by the trichomes in sunflower leaves and likely is partly secreted. Observation of brown accumulations in the tips of multi-cellular trichomes in this work suggested also the possibility of excretion of Mn containing compounds by leaf trichomes (Fig. 5). It has been recognized that trichomes of some plant species act as salt glands which accumulate solutes from adjacent cells and secrete them onto the outer surfaces of the plants [40]. In some cases (e.g. *Atriplex sp*.) the mechanism of secretion has been studied in some detail, but in many species careful studies have not been conducted. In most studies, attention has focused on secretion of sodium chloride, though it has been shown that the composition of secretions from *Tamarix aphylla* depended on the composition of the nutrient solution [4].

Tracheids around the leaf areoles showed dark discolorations in Mn treated plants with a distinct autofluorescence effect indicating likely the accumulations of phenolics. Lack of such accumulations in the midrib and its branches, however, should be explained. In dicotyledonous leaves, there are considerable different-

ces in the anatomical features between large and small veins. The large and medium-sized veins contain vessels and sieve tubes, but in the smallest veins the tracheary elements are tracheids with annular and spiral wall thickenings. In the small or micro veins the parenchyma cells in contact with the sieve elements and tracheary elements constitute transfer cells. Some of these cells are believed to be concerned with short distance translocation between mesophyll and sieve elements and others with an exchange of solutes between the xylem and phloem. Moreover, the small veins that form a network between the larger veins, are usually situated in the outermost layer of the spongy mesophyll which borders the palisade cells [16]. All of these differences could be involve in different response to excess Mn between large and small veins. Uneven distribution of peroxidases, different chemical form of Mn accumulation in the terminal veins and in the interface of xylem and phloem in thin veins and its possible association with phenolics in this part similar with species such as cowpea [44] are of possible explanations. On the other hand, because the upward movement of elements in the xylem is driven predominantly by transpiration [32], an increase in Mn concentration from the midrib to the small veins is expected. Therefore, more Mn may accumulate at the end of the transpiration stream which likely associated with accumulation of phelonic compounds in this part as shown for cowpea [44]. An increase in Mn concentration from the midrib to the marginal part was observed within a leaf of *Phytolacca acinosa*, a Mn hyperaccumulator species [47]. A transpiration-driven distribution pattern of Mn in shoot may also explain the observed difference between mature and younger leaves in the expression of symptoms.

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