

# INFLUENCE OF MANGANESE DEFICIENCY ON METAL ION UPTAKE, ANTIOXIDANT DEFENSE MECHANISM AND LIPID PEROXIDATION LEVELS IN *MENTHA PIPERITA* LEAVES

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We investigated the antioxidant defense mechanism, metal uptake and lipid peroxidation (LPO) levels at different leaf positions in *Mentha piperita* L. grown in  $Mn^{2+}$ -deficient and control conditions. Under manganese deficiency the activity of superoxide dismutase (SOD), catalase (CAT) and guaiacol peroxidase (GuaPOX) and the content of ascorbate, chlorophyll, and carotenoid under  $Mn^{2+}$  deficiency were significantly lower than in the control for all leaf positions. SOD activity correlated positively with  $Mn^{2+}$  uptake.  $Fe^{2+}$  uptake was inhibited by  $Mn^{2+}$  deficiency. During early stages of  $Mn^{2+}$  deficiency, *M. piperita* leaves showed relatively more antioxidant activity and lower LPO. Towards the final stages of the treatment period, comparatively lower SOD, CAT and GuaPOX activity and higher LPO levels accelerated the senescence process.

**Key words:** Antioxidant defense system, lipid peroxidation, *Mentha piperita*,  $Mn^{2+}$  deficiency, metal uptake.

## INTRODUCTION

Manganese ( $Mn^{2+}$ ) is an essential plant micronutrient which is involved in the activation of many enzymes in plant systems, mostly in oxidation-reduction, decarboxylation and hydrolytic reactions (Marchner, 1995a). Incorporation of  $Mn^{2+}$  by cells is essential, particularly in photosynthesis, where  $Mn^{2+}$  plays a critical role as an accumulator of positive charge equivalents in a reaction catalyzed in photosystem II (Marschner, 1995b).  $Mn^{2+}$  is also important in the synthesis of riboflavin, ascorbic acid and carotene.

Plant species differ considerably in the levels of their normal or adequate  $Mn^{2+}$  leaf concentrations (30–500 mg  $Mn^{2+}kg^{-1}$  dry mass, Clarkson, 1988). Absorption of  $Mn^{2+}$  by plant roots depends on the ability of the plants to transfer the metal across the soil-root interface and on the available amount of  $Mn^{2+}$  in nutrient (Farasova and Beinrohr, 1998). Regulation within the plant involves precisely targeted transport from the macro-level of the tissue to the micro-level of the cell and organelles. The cellular concentration of  $Mn^{2+}$  needs to be finely controlled. Any change away from the threshold level can lead

to toxic or deficiency effects (Lidon and Teixeira, 2000, Henriques, 2003, Boojar and Goodarzi, 2008, Hauck et al., 2002, Hauck et al., 2003, Lei et al., 2007).

$Mn^{2+}$  deficiency is a widespread plant nutritional disorder in agriculture. It is difficult to overcome because  $Mn^{2+}$  is rapidly oxidized when supplemented artificially in fertilizers. Selection and breeding of plant genotypes tolerant to micronutrient deficiency is a sustainable way to enhance productivity. The success of such an approach depends upon a better understanding of the mechanisms governing tolerance to nutrient deficiency. There is increasing evidence that much of the harm to plants that is due to various environmental stresses is associated with oxidative damage through direct or indirect formation of reactive oxygen species (ROS). The ROS, including superoxide ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), hydroxyl radicals ( $\cdot OH$ ) and singlet oxygen ( $^1O_2$ ), are inevitable byproducts of cell metabolism. These ROS attack lipids, proteins and nucleic acids, causing lipid peroxidation, protein

**Abbreviations:** APX – ascorbate peroxidase; CAT – catalase; GuaPOX – guaiacol peroxidase; MDA – malondialdehyde; LPO – lipid peroxidation; ROS – reactive oxygen species; 6-OHDA – 6-hydroxydopamine; SOD – superoxide dismutase; TBA – thiobarbituric acid.

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denaturation and DNA mutation (Bowler et al., 1992). Under physiological conditions the production and destruction of ROS is regulated well in the cell metabolism by the antioxidant defense system. Under stress conditions, however, the formation of these radicals can exceed the amount present under physiological conditions, thus creating oxidative stress.

Among the antioxidant enzymes, superoxide dismutase (SOD) catalyzes dismutation of the superoxide anion ( $O_2^-$ ) into hydrogen peroxide, while catalase (CAT) and peroxidases (AsA-dep and GuaPOX) detoxify  $H_2O_2$ . Other antioxidants such as ascorbate, carotenoids, polyphenols and flavonoids scavenge ROSs.

Though much work has been done on production of oxidants and antioxidants during senescence, little information is available on the actual role of oxidative stress and antioxidants in relation to the progression of leaf senescence under  $Mn^{2+}$  deficiency over time (Prochazkova et al., 2001; Li et al., 2005; Lei et al., 2007; Lehner et al., 2008).

In this study we (1) examined the activity of antioxidant enzymes (SOD, CAT, AsA-dep, GuaPOX) and the levels of chlorophyll, carotenoid, ascorbate, and membrane LPO at different leaf positions of *Mentha piperita*, (2) measured  $Mn^{2+}$ ,  $Cu^{2+}$ ,  $Zn^{2+}$  and  $Fe^{2+}$  content at leaf 6 of *M. piperita*, and (3) determined antioxidant enzyme activity and LPO levels at leaf 6 under  $Mn^{2+}$ -deficient conditions over time.

## MATERIALS AND METHODS

### PLANT CULTURE CONDITION

Seeds of *Mentha piperita* L. were disinfected with 10%  $H_2O_2$  for 20 min, washed thoroughly with distilled water and germinated between wet paper towels at 25°C in the dark for 3 days. Seedlings were grown in a growth chamber under white fluorescent light (Philips, irradiance  $750 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) with a 16 h photoperiod at 25/20°C (day/night) and  $65 \pm 5\%$  relative humidity. The seedlings were grown in Hoagland and Arnon solutions ( $1.18 \mu\text{M Mn}^{2+}$ ) until the first 8 leaves leafed out (1953). The nutrient solution contained per liter:  $\text{KNO}_3$  (1.02 g);  $\text{Ca}(\text{NO}_3)_2$  (0.492 g);  $\text{NH}_4\text{H}_2\text{PO}_4$  (0.23 g);  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.49 g);  $\text{H}_3\text{BO}_3$  (2.66 mg);  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  (1.81 mg);  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.08 mg);  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (0.22 mg);  $\text{H}_2\text{MoO}_4 \cdot \text{H}_2\text{O}$  (0.09 mg) and 0.5%  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (0.6 mL). These solutions were permanently aerated and renewed 3 to 4 times a week to minimize pH shift and nutrient depletion. The seedlings were then transferred to nutrient solution, which included  $0 \mu\text{M Mn}^{2+}$  as deficiency,  $1.18 \mu\text{M Mn}^{2+}$  as the control.

The direction of leaf positions was counted downward (from leaf position 2 to 8). At harvest,

14-day-old leaves were weighed and used for preparation of extracts for enzyme analysis. Extracts of *M. piperita* leaves were prepared for enzyme determinations.

### ENZYME ACTIVITY ASSAYS

Leaf material (1 g, without main midribs) was homogenized in 4 ml 20 mM phosphate buffer (pH 7.4) containing 50 mM  $\beta$ -mercaptoethanol. The homogenate was filtered and then centrifuged at  $15,000 \times g$  for 15 min. The supernatant was used for enzyme analysis. All operations (before enzyme determination) were performed at 0 to 4°C.  $\beta$ -mercaptoethanol was not included in the homogenization buffer system for determination of GuaPOX activity and LPO levels. Superoxide dismutase (EC 1.15.1.1) activity was determined according to Crosti et al. (1987). One unit of enzyme activity is defined as 50% inhibition of 6-hydroxydopamine (6-OHDA) autoxidation under assay conditions. Different forms of SOD were identified using KCN and/or  $H_2O_2$ . KCN inhibits Cu/ZnSOD but does not affect MnSOD nor FeSOD, while  $H_2O_2$  inactivates Cu/ZnSOD and FeSOD without affecting MnSOD (Yu and Rengel, 1999). Ascorbate-dependent peroxidase activity (EC 1.11.1.11) was measured according to Nakano and Asada (1981) by monitoring the rate of ascorbate oxidation at 290 nm ( $E = 2.8 \text{ mM}^{-1}\text{cm}^{-1}$ ). The reaction mixture contained 25 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 1 mM  $H_2O_2$ , 0.25 mM AsA and the enzyme sample. There was no change in absorption in the absence of AsA in the test medium. For guaiacol-dependent peroxidase (EC 1.11.1.7) activity, the reaction mixture contained 25 mM phosphate buffer (pH 7.0), 0.05% guaiacol, 10 mM  $H_2O_2$  and enzyme. Activity was determined by the increase in absorbance at 470 nm due to guaiacol oxidation ( $E = 26.6 \text{ mM}^{-1}\text{cm}^{-1}$ ) (Nakano and Asada, 1981). Catalase (EC 1.11.1.6) activity was assayed in a reaction mixture containing 25 mM phosphate buffer (pH 7.0) 10.5 mM  $H_2O_2$  and enzyme in 25 mM phosphate buffer (pH 7.0). Decomposition of  $H_2O_2$  was followed at 240 nm ( $E = 39.4 \text{ mM}^{-1}\text{cm}^{-1}$ ) (Aebi, 1983).

### ANALYTICAL METHODS

The concentrations of the metals were measured by inductively coupled plasma spectroscopy (ICP Model-8410, Labtam, Australia). To determine metal content in *M. piperita*, 1 g wet weight of leaves was digested in 2 ml nitric acid followed by 2 ml perchloric acid. All glassware and apparatus were washed with ultrapure water and 0.1 M  $\text{HNO}_3$  before use. Concentrations of chlorophyll ( $a + b$ ) and carotenoids in leaves were measured as described by Lichtenthaler and Wellburn (1983) after extraction

TABLE 1. Activity of SOD, CAT and POD enzymes, levels of LPO, Chl and Car, and Chl/Car ratio, at different leaf positions of *M. piperita* on day 14.

	Leaf Position					LSD (0.05)
	Mn <sup>2+</sup> (μM)	2	4	6	8	
SOD (IU/mg)	1.18*	38.1	41.5	48.0	41.1	1.8
	0	9.3	9.6	19.3	10.3	
CAT(IU/mg)	1.18	14.7	15.6	16.0	14.0	0.8
	0	4.8	5.2	5.6	2.7	
AsA (mM)	1.18	10.6	12.3	14.7	11.2	2.0
	0	9.1	10.4	12.5	10.7	
APX (IU/mg)	1.18	3.2	3.6	4.9	2.6	0.3
	0	4.5	4.6	6.5	4.5	
GuaPOX (IU/mg)	1.18	7.4	7.8	7.9	7.0	0.2
	0	1.0	1.3	1.5	0.9	
LPO (nmol MDA/g)	1.18	5.6	5.2	4.6	4.8	1.3
	0	8.0	8.0	7.5	9.0	
Chl (μg cm <sup>-2</sup> )	1.18	24.3	25.2	26.5	24.1	3.0
	0	18.3	19.0	22.0	17.9	
Car (μg cm <sup>-2</sup> )	1.18	3.0	3.1	3.3	3.0	0.3
	0	2.5	2.6	2.9	2.8	
Chl / Car	1.18	8.0	8.1	8.0	8.0	0.6
	0	7.3	7.3	7.7	6.4	

\* Control

with 80% acetone. The absorbance of the pigment extract was measured at 470, 646 and 663 nm. The content of Chl *a*, Chl *b* and carotenoids were estimated with the experimental equations described by Lichtenthaler and Wellburn (1983). Ascorbate concentrations were immediately determined with 2,4-dinitrophenylhydrazine (Nino and Shaw, 1976). The sample absorbance was measured at 460 nm. Lipid peroxidation was estimated based on thiobarbituric acid (TBA) reactivity. Samples were evaluated for malondialdehyde (MDA) production using a spectrophotometric assay for TBA. The extinction coefficient of 153 mM<sup>-1</sup>cm<sup>-1</sup> at 532 nm for the chromophore was used to calculate the MDA-like TBA produced (Buege and Aust, 1978). Protein content was determined according to Bradford (1976) using bovine serum albumin (BSA) as standard.

### STATISTICAL ANALYSIS

The Tukey test for multiple comparisons was used to verify the significance of results. Values are means of three separate experiments. Pearson's correlations were used to compare substrates and enzymes.

### RESULTS

As seen in Table 1, SOD, CAT and PODs activity and AsA levels reached maximum at leaf 6 and decreased beyond that (leaf position 8) ( $p < 0.05$ ). There was a very close positive relationship between Mn<sup>2+</sup> concentration and the activity of SOD ( $r = 0.805$ ,  $p < 0.05$ ). CAT and GuaPOX activity, AsA, chlorophyll, and carotenoid content in Mn<sup>2+</sup>-deficient conditions were significantly lower, while APX activity was higher than in the control ( $p < 0.05$ ). Chlorophyll/carotenoid ratios in Mn<sup>2+</sup>-deficient conditions were changed, between  $7.3 \pm 0.1$  and  $6.4 \pm 0.1$ . Membrane LPO levels under Mn<sup>2+</sup> deficiency increased along the stem versus the control. In contrast to the variation of antioxidant enzyme activity, LPO level reached minimum at leaf 6 and increased with age.

Since enzyme activity was highest and the LPO level was at minimum in leaf 6, this position was used in further stages of the experiment.

Cu<sup>2+</sup>, Zn<sup>2+</sup>, Fe<sup>2+</sup> and Mn<sup>2+</sup> levels were assessed at leaf 6 of *M. piperita* on the 14th day. As seen in Table 2, Mn<sup>2+</sup> and Fe<sup>2+</sup> uptake by *M. piperita* grown in Mn<sup>2+</sup>-deficient conditions decreased by ~3.3- and ~2.8-fold, respectively. Cu<sup>2+</sup> and Zn<sup>2+</sup> levels did not change significantly with Mn<sup>2+</sup> deficiency.

TABLE 2.  $Mn^{2+}$ ,  $Fe^{2+}$ ,  $Zn^{2+}$  and  $Cu^{2+}$  concentrations in leaf 6 of *M. piperita*. Each data point represents the mean of three replicates

$Mn^{2+}$ (mM)	$Mn^{2+}$	$Fe^{2+}$	$Zn^{2+}$	$Cu^{2+}$
	(mg g <sup>-1</sup> DW)			
0	26.6	32.4	42.0	14.0
0,12	35.4	42.8	43.6	14.5
1.18*	87.8	92.8	45.2	15.0
LSD (0.05)	1.9	1.6	2.7	2.3

\* Control

Changes in SOD activity in leaf 6 of *M. piperita* plants grown with  $Mn^{2+}$  supply decreasing from 1.18 to 0  $\mu M$  were assessed with respect to time. SOD activity decreased sharply with  $Mn^{2+}$  deficiency. SOD activity in control conditions (1.18  $\mu M$ ) did not change significantly over time ( $p > 0.05$ ) (Fig. 1). In the treatments with 0.118–0  $\mu M$   $Mn^{2+}$ , SOD activity in *M. piperita* leaves dropped continuously during days 1 to 17, and the reduction became significant between days 1 and 6. There was a positive correlation between SOD activity and  $Mn^{2+}$  concentration ( $r = 0.734$ ,  $p < 0.05$ ).

CAT activity showed a similar trend in leaf 6 of *M. piperita* in the 0.590  $\mu M$   $Mn^{2+}$  treatment ( $p > 0.05$ ) (Fig. 2). It progressively decreased under different  $Mn^{2+}$  concentrations up to day 12 and did not differ significantly between treatments thereafter. CAT activity and  $Mn^{2+}$  concentration correlated positively with time ( $r = 0.545$ ,  $p < 0.05$ ).

As seen from Figure 3, APX activity under  $Mn^{2+}$  deficiency reached maximum, exceeding the level in the control, between days 10 and 12. APX activity was below control levels in the rest of the treatment period ( $p > 0.001$ ). GUAPOX activity in  $Mn^{2+}$ -deficient conditions was below that of the control throughout the treatment period (Fig. 4). It decreased up to day 12, and did not change significantly thereafter ( $p > 0.001$ ). GUAPOX activity correlated positively with  $Mn^{2+}$  concentration ( $r = 0.844$ ,  $p < 0.05$ ).

Figure 5 shows that LPO activity, unlike SOD, CAT, GUAPOX activity, correlated negatively with  $Mn^{2+}$  concentration ( $r = -0.797$ ,  $p < 0.05$ ). LPO levels in leaf 6 decreased up to day 6 and increased continuously thereafter in all the manganese-deficiency treatments ( $p < 0.001$ ).

## DISCUSSION

Manganese is a transition metal, and  $Mn^{2+}$  stress is a major factor limiting plant growth (Hauck et al., 2003; Shi et al., 2005). The molecular, physiological

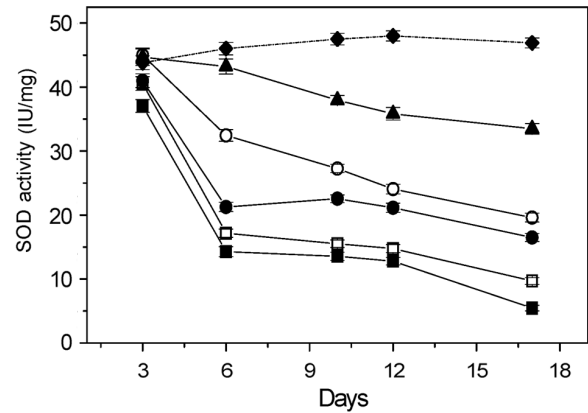


Fig. 1. SOD activity in leaf 6 of *M. piperita* in  $Mn^{2+}$  deficiency conditions over time: (-■-) 0  $\mu M$ , (-□-) 1.18  $10^{-2}$   $\mu M$ , (-●-) 2.36  $10^{-2}$   $\mu M$ , (-○-) 1.18  $10^{-1}$   $\mu M$ , (-▲-) 5.9  $10^{-1}$   $\mu M$ , and (-◆-) 1.18  $\mu M$   $Mn^{2+}$ . Each data point represents the mean of three replicates.

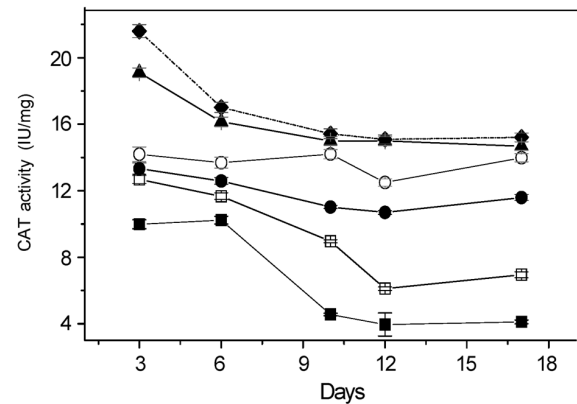
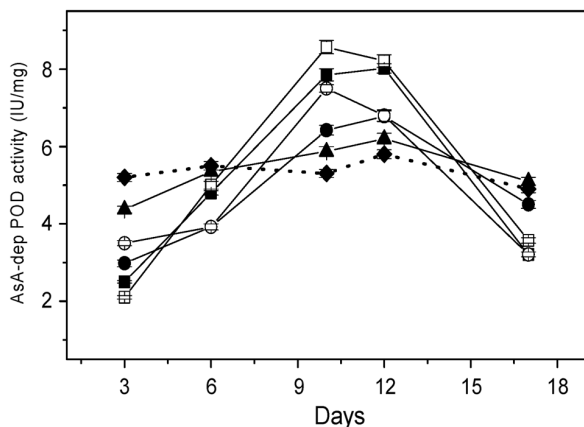


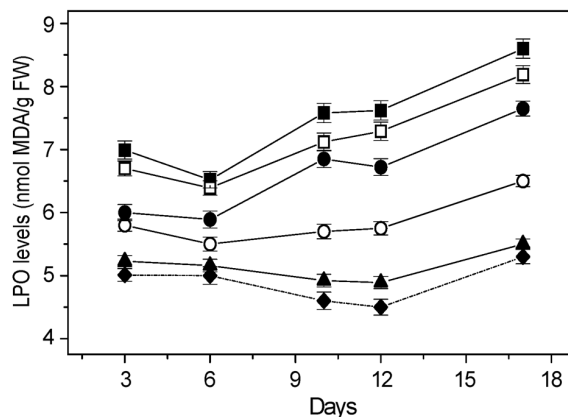
Fig. 2. CAT activity in leaf 6 of *M. piperita* in  $Mn^{2+}$  deficiency conditions over time: (-■-) 0  $\mu M$ , (-□-) 1.18  $10^{-2}$   $\mu M$ , (-●-) 2.36  $10^{-2}$   $\mu M$ , (-○-) 1.18  $10^{-1}$   $\mu M$ , (-▲-) 5.9  $10^{-1}$   $\mu M$ , and (-◆-) 1.18  $\mu M$   $Mn^{2+}$ . Each data point represents the mean of three replicates.

and genetic mechanisms of  $Mn^{2+}$  tolerance in plants are insufficiently known (Santandrea et al., 2000).

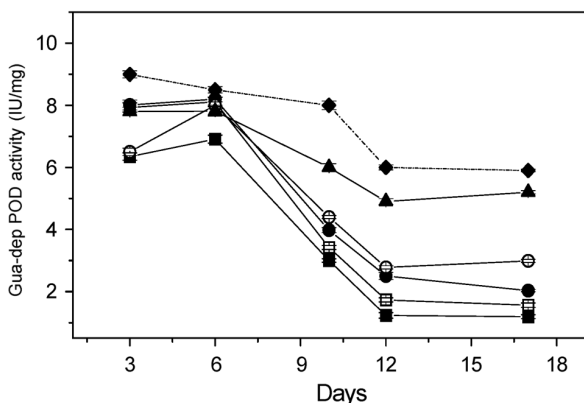
In this study we found that SOD activity correlated positively with  $Mn^{2+}$  concentration. These results were coherent with the presence of MnSOD in *M. piperita* leaves because SOD activity was insensitive to both KCN and  $H_2O_2$  in the crude extract. The MnSOD activity of *M. piperita* leaves was 50% of total SOD activity. MnSOD and FeSOD activity decreased, while Cu/Zn SOD activity remained unaffected by  $Mn^{2+}$  deficiency (data not shown). In higher plants, MnSOD is widely distributed in chloroplasts. This may imply that leaf chloroplasts/mitochondria are the major site of superoxide radical formation under stress condi-



**Fig. 3.** APX activity in leaf 6 of *M. piperita* in  $Mn^{2+}$  deficiency conditions over time: (-■-) 0  $\mu M$ , (-□-)  $1.18 \cdot 10^{-2}$   $\mu M$ , (-●-)  $2.36 \cdot 10^{-2}$   $\mu M$ , (-○-)  $1.18 \cdot 10^{-1}$   $\mu M$ , (-▲-)  $5.9 \cdot 10^{-1}$   $\mu M$ , and (-◆-)  $1.18 \mu M Mn^{2+}$ . Each data point represents the mean of three replicates.



**Fig. 5.** LPO levels in leaf 6 of *M. piperita* in  $Mn^{2+}$  deficiency conditions over time: (-■-) 0  $\mu M$ , (-□-)  $1.18 \cdot 10^{-2}$   $\mu M$ , (-●-)  $2.36 \cdot 10^{-2}$   $\mu M$ , (-○-)  $1.18 \cdot 10^{-1}$   $\mu M$ , (-▲-)  $5.9 \cdot 10^{-1}$   $\mu M$ , and (-◆-)  $1.18 \mu M Mn^{2+}$ . Each data point represents the mean of three replicates.



**Fig. 4.** GuaPOX activity in leaf 6 of *M. piperita* in  $Mn^{2+}$  deficiency conditions over time: (-■-) 0  $\mu M$ , (-□-)  $1.18 \cdot 10^{-2}$   $\mu M$ , (-●-)  $2.36 \cdot 10^{-2}$   $\mu M$ , (-○-)  $1.18 \cdot 10^{-1}$   $\mu M$ , (-▲-)  $5.9 \cdot 10^{-1}$   $\mu M$ , and (-◆-)  $1.18 \mu M Mn^{2+}$ . Each data point represents the mean of three replicates.

tions in *Mentha piperita*, and consequently that MnSOD is the major isoform responsible for superoxide radical scavenging during stress (Lidon and Teixeira, 2000).

We found that chlorophyll content was significantly reduced by  $Mn^{2+}$  deficiency, indicating the critical role of  $Mn^{2+}$  ions as a cofactor in photosynthetic light-dependent reactions. Lower chlorophyll/carotenoid ratios are an indicator of senescence, stress and damage to the plant and the photosynthetic apparatus, expressed by faster breakdown of chlorophylls than carotenoids (Lichtenthaler and Buschmann, 2001). Ascorbate has a central role in the detoxification of ROS in plant cells under various stress conditions. *M. piperita* leaves responded to  $Mn^{2+}$  deficiency with a remarkable decrease in ascorbate content. This

decrease might be explained by increases in lipid peroxidation (Tab. 1).

The molecular details of  $Mn^{2+}$  metabolism and biochemical consequences of deficiency are still under investigation in relation to Fe metabolism. In our study,  $Fe^{2+}$  content in leaf 6 under  $Mn^{2+}$  deficiency decreased as much as 2.9-fold (Tab. 2). Allen et al. (2007) reported that  $Mn^{2+}$  deficiency induces secondary Fe deficiency, probably as a result of reduction of uptake of Fe (catalyzing the Haber Weiss reaction) in order to avoid more oxidative stress due to the reduced activity of MnSOD. The loss of MnSOD and FeSOD activity presumably is a consequence of the deficit of manganese (and secondary Fe deficiency), which is a necessary cofactor. Iron is a cofactor of important antioxidant enzymes such as CAT and PODs, which are involved in maintaining the balance of the redox state. Despite the significant decline in the activity of SOD, CAT and GuaPOX, *M. piperita* leaves showed irregular changes in APX activity in response to  $Mn^{2+}$  deficiency. The irregular changes in APX activity might indicate preferential synthesis/activation of this enzyme by *Mentha* plants to counteract oxidative stress. The decreases of MnSOD and FeSOD activity mentioned above were not compensated by increased CAT and GuaPOX activity, resulting in an increase of lipid peroxidation in  $Mn^{2+}$ -deficient conditions (Tab. 1). This situation might be attributed to a serious imbalance in ROS antioxidant defense systems.

During the treatment period, SOD, CAT and GuaPOX activity correlated positively with decreasing  $Mn^{2+}$  concentration ( $r = 0.922$ ,  $r = 0.575$  and  $r = 0.630$ ) (Figs. 1–5). Only APX activity showed an increasing trend during the treatment period. The different affinities of APX ( $\mu M$  range) and CAT (mM range) for  $H_2O_2$  suggest that they belong to two dif-

ferent classes of H<sub>2</sub>O<sub>2</sub>-scavenging enzymes (Mittler, 2002). APX might be responsible for fine modulation of ROS, whereas CAT might be responsible for the removal of excess ROS during stress.

The balance between SOD and CAT or PODs activity in cells is crucial in setting the steady-state level of superoxide radical and hydrogen peroxide (Bowler, 1997). During early stages of Mn<sup>2+</sup> deficiency, *Mentha piperita* leaves showed relatively more antioxidant activity and lower lipid peroxidation levels. Towards the final stages of the treatment period, comparatively lower SOD, CAT and GUAPOX activity and higher LPO levels accelerated the senescence process.

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