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Changes in Chlorophyll-Carotenoid Contents, Antioxidant Enzyme Activities and Lipid Peroxidation Levels in Zn-Stressed *Mentha pulegium*

Nilgün CANDAN
Chemistry Department, Dokuz Eylül University, Faculty of Art and Science, Kaynaklar, Buca, 35160 İzmir-TURKEY
Leman TARHAN*
Chemistry Department, Dokuz Eylül University, Faculty of Education, Buca, 35150 İzmir-TURKEY
e-mail: leman.tarhan@deu.edu.tr

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The relationships between chlorophyll-carotenoid contents, the antioxidant enzyme activities, such as superoxide dismutase (SOD), catalase (CAT), ascorbate-dependent peroxidase (AsA-dep POD) and guaiacol-dependent peroxidase (Gua-dep POD), and lipid peroxidation (LPO) levels were investigated in leaves at different positions of *M. pulegium* grown in nutrient medium containing 0-1 μM Zn. The maximum chlorophyll-carotenoid content's SOD and CAT activities and the minimum LPO level were observed in leaf 6 at 7.6 $10^{-2}\mu$M Zn (as control). While chlorophyll-carotenoid contents in the absence of Zn were lower than those of the control, SOD and CAT activities significantly increased with decreasing Zn concentration. With excess Zn, chlorophyll-carotenoid content exhibited a trend similar to that of the control. However, SOD, CAT and Gua-dep POD activities and also AsA-dep POD activities after leaf position 4 were inhibited by excess Zn. Absence of Zn in the nutrient medium caused significantly higher LPO levels than in the control. Excess Zn showed its toxic effects after leaf position 4. The effects of decreasing Zn concentrations in the range 7.67-1.8 $10^{-2}\mu$M on the same parameters were also investigated with respect to time. The sharp decreases in antioxidant enzyme activities caused increasing LPO levels after the 12^{th} day. According to the results, the highest antioxidative capacity of *M. pulegium* is in the range 0.38-7.67 10^{-2} μM, which is the optimum for preventing Fe^{2+}-initiated lipid peroxidation.

Key Words: Antioxidant Enzymes, Chlorophyll-Carotenoid, *Mentha pulegium*, LPO, Zn

Introduction

Zinc is one of the micronutrients essential for the normal growth and development of plants, as it is known to be required in several metabolic processes[1]. The presence of zinc at higher concentrations retards the

*Corresponding author

growth and development of plants, by interfering with certain important metabolic processes[2]. At the same time, zinc is important for maintaining the membrane structure and functions. In the roots of Zn-deficient plants leakage of solutes like K^+, sugars and amino acids is increased[3]. These effects of zinc were proposed to be a result of an excessive generation of reactive oxygen species (ROS)[4]. In order to effectively eliminate ROS ($O_2^{\cdot-}$, $HO^{\cdot}$, O_2^1, H_2O_2) an antioxidative system consisting of low-molecular-weight antioxidants (such as ascorbate and carotenoids) and protective enzymes that operate in the following way. Superoxide radicals are scavenged by superoxide dismutase (SOD, EC 1.15.1.1), while the resulting H_2O_2 can be detoxified by ascorbate-dependent peroxidase (AsA-dep POD, EC 1.11.1.11), guaiacol-dependent peroxidase (Gua-dep POD, EC 1.11.1.7) and catalase (CAT, EC 1.11.1.6). Under optimal conditions, the production and destruction in ROS is regulated well in the cell metabolism. Increases in ROS as a consequence of various environmental stresses result in oxidative stress. Environmental stresses, such as heavy metal toxicity, chilling, drought, high light intensity and air pollutants are directly or indirectly associated with oxidative stress. Other stress factors, such as mineral nutrient deficiencies, can also modulate the activities of antioxidative enzymes[5–8]; therefore oxidative stress is a component of micronutrient deficiency.

Because zinc deficiency and toxicity have important effects in the antioxidative defence system, in the present study the Zn tolerance capacity of *M. pulegium* leaves was examined. We also studied the response of the content of chlorophyll-carotenoid, $O_2^{\cdot-}$ and H_2O_2 scavenging enzymes together with the levels of lipid peroxidation in leaf extracts of *M. pulegium* subjected to different concentrations of zinc deficiency with respect to time.

Experimental

Plant Growth Conditions

Seeds of *Mentha pulegium* L. were disinfected with 10% H_2O_2 (v/v) for 20 min and washed thoroughly with distilled water and germinated between wet paper towels at 25°C in darkness for 3 d. Seedlings were transferred to Hoagland nutrient solutions with varied Zn concentrations[9]. These solutions were permanently aerated and renewed three to four times a week to minimize pH shift and nutrient depletion. The control medium contained 7.67 $10^{-2}\mu$M Zn. Plants were grown in a growth chamber (16 h light/8 h dark) with white fluorescent light (Philips, India) at a light intensity of 150 μmol m^{-2} s^{-1}, day/night temperature of 25/20°C and 65 ($\pm$5)% relative humidity. At harvest 14-day-old leaves were weighed and used in the preparation of extracts for enzyme analysis. Five separate experiments were conducted during the growing period of Zn-deficient *M. pulegium*.

Leaf Positions

Seedlings were selected for experiments based on their uniform appearance, in terms of the average height of the plants, the total number of leaves present on each plant and the size of leaves. The leaves at the shoot apex were considered leaf position 2 (leaf 2). Leaves 4, 6 and 8 were labelled in all seedlings, counting down from the top of the plants.

Enzyme Determinations

Extracts of *M. pulegium* organs were prepared for enzyme determinations. Usually 1 g of leaf (without the main midribs) material was homogenized in 4 ml 20 mM phosphate buffer (pH 7.4) containing 50 mM β-Mercaptoethanol. The homogenate was filtered and then centrifuged at 15,000 g for 15 min. The supernatant was used for enzyme analysis. All operations (until enzyme determinations) were carried out at 0 to 4°C. β-Mercaptoethanol was not included in homogenizing buffer for the determinations of Gua-dep POD activity or lipid peroxidation (LPO) levels.

SOD Activity Assay

The SOD assay was based on the inhibitory effects of SOD on the spontaneous autoxidation of 6-hydroxidopamine[10]. The autoxidation rate of 6-OHDA 0.4 mM in 0.1 M phosphate buffer pH 7.4, which was saturated by air-O_2 (8.2 mg/l), was determined by observing the absorbance changes at 490 nm over 15 s intervals at 25°C. The SOD activity assay was carried out by adding the amount of enzyme solution required to half the initial absorbance value of 6-OHDA autoxidation at 90 s. One IU is the amount of SOD required to inhibit the initial rate of 6-hydroxydopamine autoxidation by 50%.

AsA-dep POD Activity Assay

AsA-dep POD activity was measured according to Nakano and Asada by monitoring the rate of ascorbate oxidation at 290 nm ($E = 2.8\ mM^{-1}\ cm^{-1}$)[11]. 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 at 25°C. No change in absorption was found in the absence of AsA in the test medium.

For AsA-dep POD, 1 IU represents the amount of enzyme catalysing the conversion of 1 μmol of substrates per minute.

Gua-dep POD Activity Assay

For the measurement of Gua-dep POD activity, the reaction mixture contained 25 mM phosphate buffer (pH 7.0), 0.05% guaiacol, 10 mM H_2O_2 and the enzyme sample. Activity was determined by the increase in absorbance at 470 nm due to guaiacol oxidation at 25°C ($E = 26.6\ mM^{-1}\ cm^{-1}$)[11].

For PODs, 1 IU represents the amount of enzyme catalysing the conversion of 1 μmol of substrates per minute.

CAT Activity Assay

CAT activity was assayed in a reaction mixture containing 25 mM phosphate buffer (pH 7.0), 10.5 mM H_2O_2 and enzyme. The decomposition of H_2O_2 was followed at 240 nm ($E = 39.4\ mM^{-1}\ cm^{-1}$)[12]. One IU was the amount of the enzyme decomposing 1 μmol H_2O_2 per minute at 25°C.

Analytical Methods

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

at 532 nm of 153 mM^{-1} cm^{-1} for the chromophore was used to calculate the MDA-like TBA produced[13].

Concentrations of Chl (a + b) and carotenoids were measured as described by Lichtenthaler and Welburn after extraction with 80% acetone[14].

The protein content was determined by the method of Bradford using bovine serum albumin (BSA) as standard[15].

Statistical Analysis

A Tukey test, one of the multiple comparisons, was used for statistical significance analyses. The values are the mean of three separate experiments. In addition, a comparison was made with Pearson correlation for each substrate and/or enzyme.

Results

Variations Chlorophyll-Carotenoid Contents, Antioxidant Enzyme Activities, and LPO Levels in Leaves at Different Positions of *M. pulegim* under Zn Stress

The effects of Zn as a phytotoxic micronutrient on chlorophyll-carotenoid contents and antioxidant enzyme activities were investigated in different leaf positions of *M. pulegium* grown in the presence of 1.0 μM and 7.67 10^{-2} μM Zn (as control) and in the absence of Zn. As can be seen from Figures 1 A-B, similar variations in SOD activities were obtained in all leaf positions of *M. pulegium* grown in the presence of 1 μM Zn and 7.67 10^{-2} μM ($p > 0.01$) and they were higher than those in the absence of Zn ($p < 0.01$).

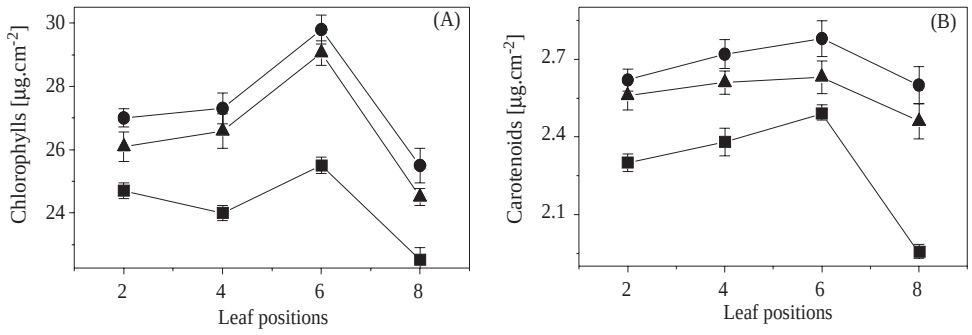

Figure 1. Content of total chlorophylls (A) and total carotenoids (B) in leaves at different positions of 14-day-old *M. pulegium* seedlings grown in control (0.0767 μM Zn^{2+}) (-▲-), 1 μM Zn^{2+} (-●-) and in the absence of Zn^{2+} (-■-).

SOD activities and chlorophyll-carotenoid contents reached maximum values at leaf 6 for all Zn concentrations and decreased with age. SOD and CAT activities in leaves grown in the absence of Zn were higher ($p < 0.01$), while they were lower in the presence of 1μ M Zn as compared with the control ($p < 0.01$) (Figures 2 A-B).

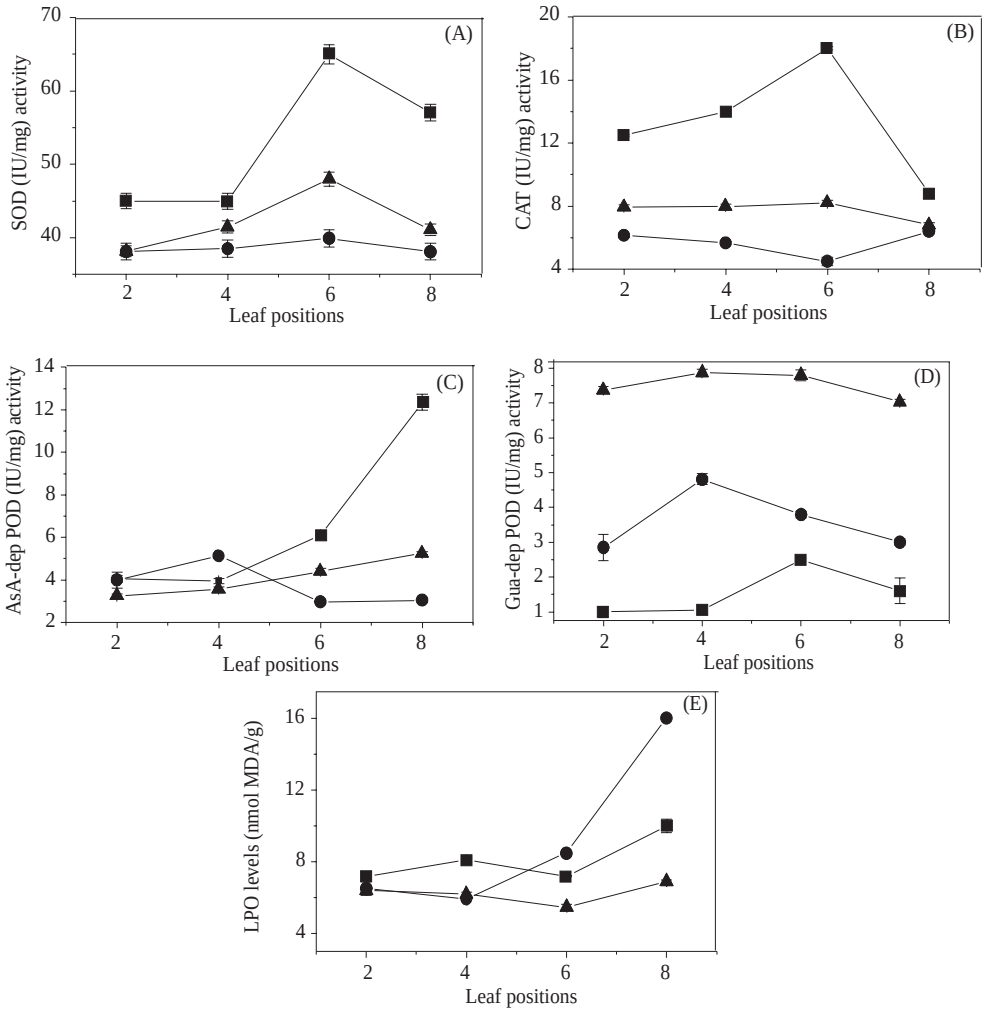

Figure 2. Variations in SOD (A), CAT (B), AsA-dep POD (C), Gua-dep POD activities (D) and LPO levels (E) in leaves at different positions of 14-day-old *M. pulegium* seedlings grown in control (0.0767 μM Zn^{2+}) (-▲-), 1 μM Zn^{2+} (-●-) and in the absence of Zn^{2+} (-■-).

AsA-dep POD activities in the control group increased along the stems of *M. pulegium* and then decreased slightly. The activities under both stress conditions showed different activity values from those of the control up to leaf 6 and increased above the control level in leaf 8 (Figure 2C) ($p < 0.01$). However, variations in Gua-dep POD activities under both stress conditions were lower than those of the control (Figure 2D).

As can be seen from Figure 2E, the leaf LPO levels in the control and in the absence of Zn, which were higher than the control, did not show significant differences up to leaf position 6 and increased afterwards. However, significant increases were observed in the presence of 1 μM Zn after leaf 4 ($p < 0.01$).

Leaf 6 of the control group, which had the maximum chlorophyll-carotenoid content, antioxidant enzyme activities and the minimum LPO level, was used in further experimental stages.

The Effect of Zn Deficiency on Chlorophyll-Carotenoid Contents of *M. pulegium* Leaves in Zn Deficiency with Respect to Time

Zn deficiency resulted in inhibition of leaf expansion and total leaf area when compared with the control. The first sign of zinc deficiency is interveinal chlorosis in older *M. pulegium* leaves, starting at the top and margins. Zn deficiency also distorted the appearance of the *M. pulegium* leaves. As can be seen from Figure 3, chlorophyll-carotenoid contents decreased significantly between days 9 and 22 and showed a positive correlation with decreasing Zn concentration over the treatment period ($r = 0.504$, $r = 0.599$; $p < 0.01$).

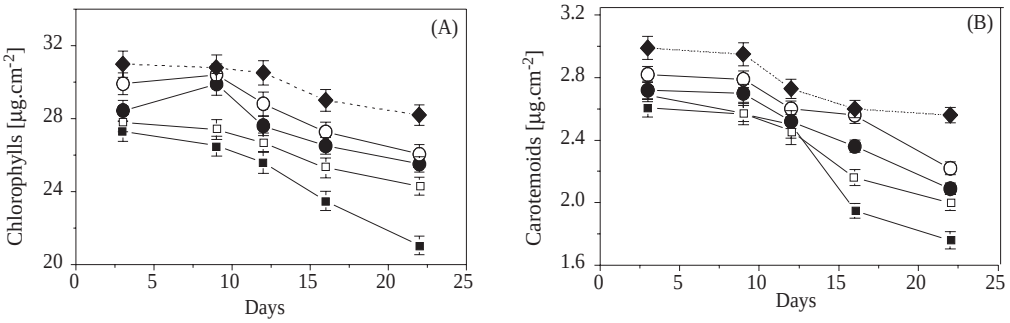

Figure 3. Variations in total chlorophyll (A) and carotenoid (B) in Zn deficiency conditions with respect to time in leaf 6 of *M. pulegium* [Zn^{2+}], μM: (-■-) 1.9 10^{-3}, (-□-) 3.8 10^{-3}, (-● -) 7.6 10^{-3}, (-◯-) 1.5 10^{-2} and control groups (-♦-) 7.67 10^{-2}.

The Effect of Zn Deficiency on Antioxidant Enzymes Activities and LPO Levels in *M. pulegium* Leaves

SOD activity variations in *M. pulegium* leaves (leaf 6) under different Zn deficiency conditions were investigated with respect to time (Figure 4).

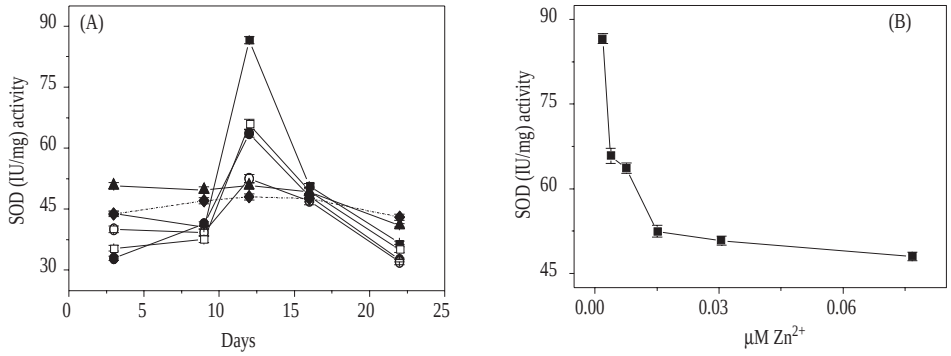

Figure 4. SOD activity variations (A) in Zn^{2+} deficiency conditions with respect to time in leaf 6 of *M. pulegium* [Zn^{2+}], μM: (-■-) 1.9 10^{-3}, (-□-) 3.8 10^{-3}, (-● -) 7.6 10^{-3}, (-◯-) 1.5 10^{-2}, (-▲-) 3.06 10^{-2} and (-♦-) 7.67 10^{-2} (B) SOD activities depending on Zn^{2+} concentrations on day 12.

According to our results, SOD activity of the control increased slightly over the 16 days of the treatment period and decreased slightly afterwards ($p < 0.01$). The variations in maximum activity values

depending on the decreasing of Zn from 7.67 10^{-2} to 1.9 $10^{-3}\mu$M increased significantly from 48.00 ± 0.86 to 86.57 ± 0.75 IU/mg on day 12 ($p < 0.01$), but in general the activities were below the control level during the treatment period.

CAT activities, which were higher than the control, did not show significant differences with respect to time, but increased to the maximum values on day 12 and then decreased below the control levels. CAT activities of the control group decreased over the treatment period, except for between days 16 and 23 (Figure 5).

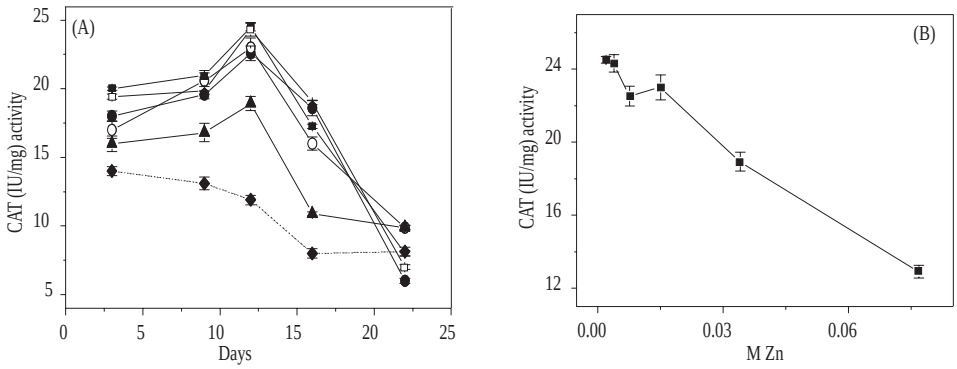

Figure 5. CAT activity variations (A) in Zn^{2+} deficiency conditions with respect to time in leaf 6 of *M. pulegium* [Zn^{2+}], μM: (-■-) 1.9 10^{-3}, (-□-) 3.8 10^{-3}, (-● -) 7.6 10^{-3}, (-◯-) 1.5 10^{-2}, (-▲-) 3.06 10^{-2} and (-◆-) 7.67 10^{-2} (B) CAT activities depending on Zn^{2+} concentrations on day 12.

Figure 6 shows that AsA-dep POD activities for the control group increased during the first 12 days and then decreased. The activities in the range 0.19-1.5 $10^{-2}\mu$M Zn concentrations were higher than the control level for 12 days and then decreased sharply below the control level ($p < 0.01$).

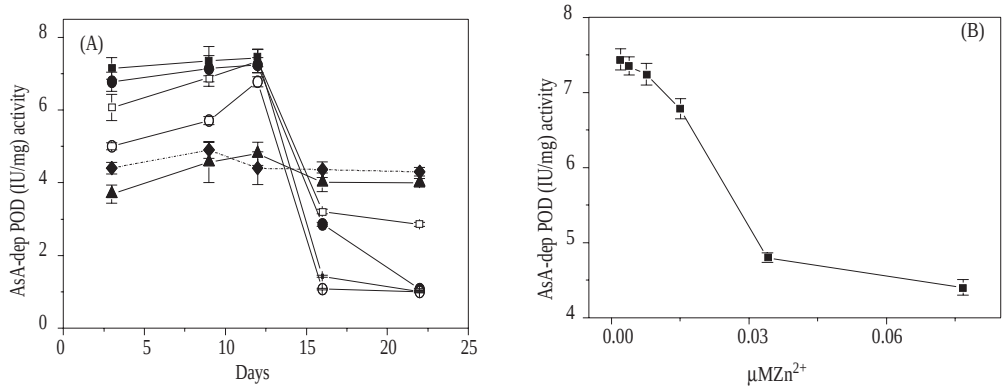

Figure 6. AsA-dep POD activity variations (A) in Zn^{2+} deficiency conditions with respect to time in leaf 6 of *M. pulegium* [Zn^{2+}], μM: (-■-) 1.9 10^{-3}, (-□-) 3.8 10^{-3}, (-●-) 7.6 10^{-3}, (-◯-) 1.5 10^{-2}, (-▲-) 3.06 10^{-2} and (-◆-) 7.67 10^{-2} (B) AsA-dep POD activities depending on Zn^{2+} concentrations on day 12.

The Gua-dep POD activities in the control group were higher than under Zn deficient conditions and they decreased slightly during the treatment period (Figure 7) ($p < 0.01$). Marked decreases in Gua-dep POD activities were observed, especially with lower Zn concentrations ($p < 0.01$).

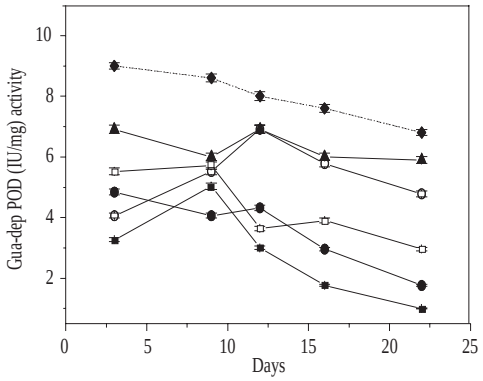

Figure 7. Gua-dep POD activity variations in Zn^{2+} deficiency conditions with respect to time in leaf 6 of *M. pulegium* [Zn^{2+}], μM: (-■-) 1.9 10^{-3}, (-□-) 3.8 10^{-3}, (-● -) 7.6 10^{-3}, (-◯-) 1.5 10^{-2}, (-▲-) 3.06 10^{-2} and (-♦-) 7.67 10^{-2}.

As can be seen from Figure 8, the LPO levels in the control and other Zn deficient plants increased with respect to time ($p < 0.01$). However, the increases in LPO levels were higher than in the control in the range 0.19-1.5 $10^{-2}\mu$M Zn.

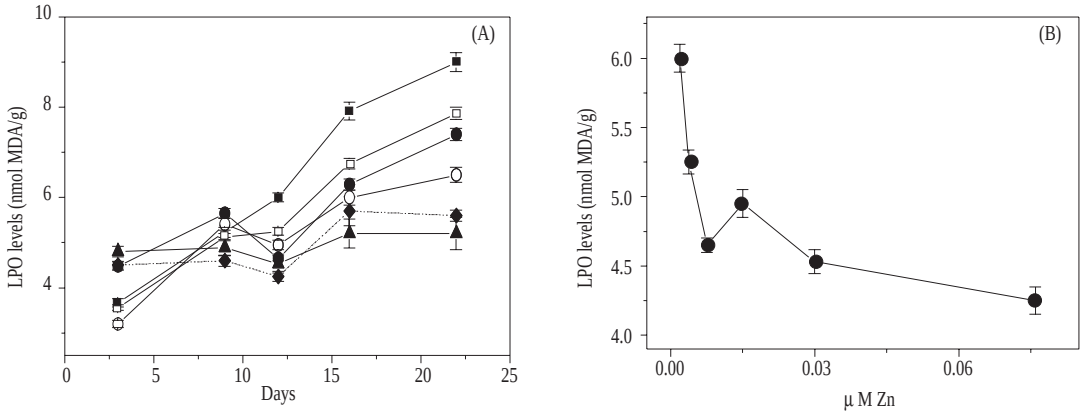

Figure 8. Levels of LPO variations (A) in Zn^{2+} deficiency conditions with respect to time in leaf 6 of *M. pulegium* [Zn^{2+}], μM: (-■-) 1.9 10^{-3}, (-□-) 3.8 10^{-3}, (-● -) 7.6 10^{-3}, (-◯-) 1.5 10^{-2}, (-▲-) 3.06 10^{-2} and (-♦-) 7.67 10^{-2}(B) LPO levels depending on Zn^{2+} concentrations on day 12.

Discussion

The role of Zn as a component of the antioxidant defence system is supported by its in vitro antioxidant function. It has been reported that Zn deficiency, both in plant and plant cell models, induces oxidative damage in cell components with alterations in antioxidant enzyme activities and contents of metabolites[16−19]. In addition, Zn, a widely spread pollutant, is readily taken up by plants and can be phytotoxic. Many studies have been devoted to the interference of Zn with a number of physiological processes associated with the normal growth and development of plants. In the current study, chlorophyll and carotenoid contents in all leaf positions of *M. pulegium* grown in the absence of Zn were found to be lower than in the control. Carotenoids are important quenchers of the singlet state of chlorophyll and singlet oxygen and the decreases

in carotenoid levels increase the possibility of ·OH formation. The lower chlorophyll concentrations can be explained by the role of Zn in protein synthesis and also reactive oxygen species are to be expected in Zn-stressed plants, leading to the peroxidation of membrane lipids and, thus, the cooxidation of chlorophyll[20]. Chlorophyll-carotenoid contents in Zn deficiency were lower than in the control over the treatment period and the falling velocity of chlorophyll-carotenoid content increased after day 10. This situation might cause the observation of sharp increases in membrane LPO levels.

SOD and CAT activities in all leaf positions of *M. pulegium* increased in the absence of Zn, while Gua-dep POD activities were not stimulated under Zn stress conditions. The increases in SOD and CAT activities in leaf positions of *M. pulegium* in the absence of Zn were not sufficient for membrane protection against the potentially increased deleterious effects of reactive oxygen species, because membrane LPO levels also increased along the stems compared with the control. Similar AsA-dep POD activity in leaf position 2-4 and lower Gua-dep POD activity in all leaf positions were observed in the absence of Zn when compared with the control. However, they cannot provide an effective contribution for the scavenging of ROS.

The effects of Zn deficiency in leaf position 6 of *M. pulegium* with respect to time have a strong and fast induction on CAT and AsA-dep POD, while there was temporary induction of SOD in a short time during the first 12 days of the treatment period. However, the increases in antioxidant enzyme activities and the membrane LPO levels also increased to above the control level with decreasing Zn concentration. The increases in LPO levels indicate that zinc might constitute an important component of the antioxidant network that protects membranes from peroxidation. It was recently reported that zinc could exert a direct antioxidant action by occupying iron or copper binding sites in lipids, proteins, and DNA[21,22]. Therefore zinc deficiency induces the intracellular accumulation of iron and copper and increased production of oxidant species[23]. Iron and copper are redox active metals that can participate in electron transfer reactions with the consequent production of oxidant species capable of peroxidizing cell components. The range 0.38-7.67 $10^{-2}\mu$M Zn in the growth medium of *M. pulegium* may be optimum for preventing Fe^{2+}-initiated lipid peroxidation of the membrane. Another reason for the increases in LPO levels may be the inhibitory effects of Zn on NADPH oxidation and NADPH-dependent $O_2^{\cdot-}$ generation documented in animal systems and in higher plants [24−27]. SOD, CAT and Gua-dep POD activities in all leaf positions of *M. pulegium* grown in medium containing excess zinc were lower than in the control and also AsA-dep POD activities were inhibited after leaf position 4. Similar to our results, Wenzel et al. found that excess Zn inhibits CAT and SOD activities in bean leaves[28]. Membrane LPO levels of *M. pulegium* leaves under excess Zn stress showed a trend similar to that of the control in leaf position 2 and 4 and then increased significantly compared to the control. These results indicated that excess Zn showed its toxic effects after leaf position 4. The very closeness of membrane LPO levels in young leaves to those in the control indicated that young leaves overcome the stress condition by effectively scavenging ROS.

The sharp decline in chlorophyll-carotenoid content and also all antioxidant enzyme activities after day 12 caused sharp increases in membrane LPO levels. The increases of the inactivation caused by superoxide anion and H_2O_2 and the decreases in protein contents may indicate that senescence process of *M. pulegium* starts after day 12 in Zn deficiency. Zn, as a protective and stabilizing component of biomembranes against reactive oxygen species, deficiency and toxicity, plays an important role in chlorophyll-carotenoid contents, activities of antioxidant enzymes and LPO levels with respect to time.

References

1. I. Cakmak and H. Marschner, **Plant nutrition-from genetic engineering to field practice**, pp. 133-137 (1993) Kluwer Academic Publishers Dordrecht.
2. S.D. Ebbs and L.V. Kochian, **J. Environ. Qual., 26,** 776-781 (1997).
3. I. Cakmak and H. Marschner, **Physiol. Plantarum, 73,** 182-186 (1988a).
4. I. Cakmak and H. Marschner, **J. Plant Physiol., 132,** 356-361 (1988b).
5. A. Del Rio, F. Sevilla, M. Gomez, J. Yanez and J. Lopez-Gorge, **Planta 1400,** 221-225 (1978).
6. I. Iturbe-Ormaetxe, J.F. Moran, C. Arrese-Igor, Y. Gogorcena, R.V. Klucas and M. Becana, **Plant Cell Environ. 18,** 421-429 (1995).
7. J.E.J. Weckx and H.M.M. Clijisters, **Physiol. Plantarum, 96,** 506-512 (1996).
8. Q. Yu, L.D. Osborne and Z. Rengel, **J. Plant Nutr., 21,** 1427-1437 (1998).
9. D.I. Arnon and D.R. Hoagland, **Soil Sci., 50,** 463 (1940).
10. N. Crosti, T. Serudei, J. Bajer and A. Serra, **J. Clin. Chem. Clin. Biochem., 25,** 265-7 (1987).
11. Y. Nakano and K. Asada, **Plant Cell Physiol., 22,** 867-880 (1981).
12. H.E. Aebi, **Methods of Enzymatic Analysis** Verlag Chemic: Deerfield Beach, FL, 3rd ed. **3**, pp. 273-286 (1983).
13. J.A. Buege and S.D. Aust, **Methods Enzymol., 52,** 464-478 (1978).
14. H.K. Lichtenthaler and A.R. Wellburn, **Biochem. Soc. Trans., 11,** 591-592 (1983).
15. M.M. Bradford, **Anal. Biochem., 72,** 248-252 (1976).
16. D. Bagchi, P.J. Vuchetich, M. Bagchi, M.X. Tran, R.L. Krohn, S.D. Ray and S.J. Stohs, **Gen. Pharmacol., 30,** 43-50 (1998).
17. P.I. Oteiza, K.L. Olin, C.G. Fraga and C.L. Keen, **J. Nutr., 125,** 823-829 (1995).
18. P.I. Oteiza, M.S. Clegg, M.P. Zago and C.L. Keen, **Free Rad. Biol. Med., 28,** 1091-1099 (2000).
19. D.J. Tate, M.V. Miceli and D.A. Newsome, **Free Rad. Biol. Med., 26,** 704-713 (1999).
20. H. Marchner and I. Cakmak, **Physiol. Plantarum, 68,** 491-496 (1986).
21. T.M. Bray and W.J. Bettger, **Free Rad. Biol. Med., 8,** 281-291 (1990).
22. S.R. Powell, **J. Nutr., 130,** 1447-1454 (2000).
23. J.M. Rogers, B. Lonerdal, L.S. Hurley and C.L. Keen, **J. Nutr., 117,** 1875-1882 (1987).
24. E.H. Jeffery, **Mol. Pharmacol., 23,** 467-473 (1983).
25. H. Suziki, M.J. Pabst and R.B. Johnson, **J. Biol. Chem., 260,** 3635-3639 (1985).
26. M. Chvapil, **Life Sci., 13,** 1041-1049 (1973).
27. M. Chvapil, **CIBA Foundation Symposium** (New Series), Excerpta Medica, Amsterdam. 163-166 (1979).
28. A.A. Wenzel and H.J. Mehlhorn, **Exp. Bot. 46,** 867-872 (1995).