Journal of Applied Botany and Food Quality 83, 103 - 109 (2010)

Chemistry Department, Faculty of Art and Science, Dokuz Eylul University, Buca, Izmir, Turkey

Alterations of the antioxidative enzyme activities, lipid peroxidation levels, chlorophyll and carotenoid contents along the peppermint (*Mentha piperita* L.) leaves exposed to copper deficiency and excess stress conditions

N. Candan, L. Tarhan*

(Received June 22, 2009)

Summary

Several physiological responses of *Mentha piperita* L. leaves at different positions along the stem were investigated under Cu^{2+} deficiency conditions and compared with excess and control. Chlorophyll and carotenoid contents and the ratio of chlorophylls to carotenoids in all leaf positions were significantly lower than those of control under Cu^{2+} deficiency. The highest decreases were determined under excess condition. Superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), ascorbate-dependent peroxidase (AsA-dep POD, EC 1.11.1.11) and guaiacol-dependent peroxidase (Gua-dep POD, EC 1.11.1.7) activities in all leaf positions in the absence of Cu^{2+} were higher than control and showed a positive correlation with each other. SOD activities under excess conditions were higher than deficiency, while all of the other enzyme activities were significantly lower than control. Co-operative functions of all these enzymes in the absence of Cu^{2+} resulted in lower lipid peroxidation levels (LPO) than control and excess conditions. Except for Gua-dep POD, all other antioxidant enzyme activities reached their maximum, while LPO level their minimum at leaf position 6. The antioxidant enzyme activities and LPO level variations were investigated with decreasing Cu^{2+} concentration ranging from 3.2×10^{-2} to 0 µM depend on the incubation time, and the results were showed an increase of 2.4-fold for SOD, 4-fold for CAT, 2-fold for AsA-dep POD and 3-fold for Gua-dep POD activities in the absence of Cu^{2+} compared to the control on the 12th day. Although enhancing antioxidant enzyme activities, LPO levels also increased approximately 2-fold compared to control in the absence of Cu^{2+} on the 12th day and its levels significantly increased with decreasing of Cu^{2+} afterwards.

Abbreviations: AsA-dep POD = ascorbate-dependent peroxidase.; CAT = catalase.; Gua-dep POD = guaiacol-dependent peroxidase.; MDA = malondialdehyde.; LPO = lipid peroxidation.; ROS = reactive oxygen species.; 6-OHDA = 6-hydroxydopamine.; SOD = superoxide dismutase.; TBA = thiobarbituric acid.

Introduction

Copper is an essential micronutrient for plants. It is a vital component of several electron transport enzymes and is involved in catalyzing the redox reactions in mitochondria and chloroplasts (SALISBURY and ROSS, 1992; MARSCHNER, 1995). The photosynthetic apparatus is particularly susceptible to this cation, resulting in a decrease of the electron transfer rates (MAKSYMIEC, 1997). Thus, plants require Cu^{2+} as an essential micronutrient for normal growth and development. When this ion is not available, plants develop specific deficiency symptoms, most of which affect leaves and reproductive organs (YRUELA, 2005). The redox properties that make Cu^{2+} an essential element also contribute to its inherent toxicity. Redox cycling between Cu^{2+} and Cu^{+} can catalyze the production of highly toxic hydroxyl radicals, with subsequent damage to DNA, lipids, proteins and other biomolecules (HALLIWELL and GUTTERIDGE, 1984). Thus, at high concentrations, Cu^{2+} can become extremely toxic causing symptoms such as chlorosis and necrosis, stunting, leaf discoloration and inhibition of root growth (VAN ASSCHE and CLIJSTERS, 1990; MARSCHNER, 1995). At the cellular level, toxicity may result from *i)* binding to sulfhydryl groups in proteins, thereby inhibiting enzyme activities or protein functions (VIARENGO, 1985; STIBOROVÁ et al., 1988); *ii)* induction of a deficiency of other essential ions; *iii)* impaired cell transport processes (YRUELA, 2005); *iv)* oxidative damage (VAN ASSCHE and CLIJSTERS, 1990).

Tolerance and protective mechanism have evolved to scavenge free radicals and peroxides generated during various metabolic pathways. These protective mechanisms include antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) and antioxidant compounds such as ascorbate, glutathione and carotenoids (ZHANG and KIRKHAM, 1996). The enzyme SOD dismutases $O_2^{\cdot-}$ to H_2O_2 and oxygen. Catalases are synthesized in a tissue specific and age dependent manner and working during the photorespiration and β-oxidation of fatty acids (LIN and KAO, 2000). Peroxidases may use ascorbate and guaiacol as electron donor, and utilize H_2O_2 in the oxidation of various inorganic and organic substrates (ASADA, 1994). The mechanisms by which Cu^{2+} induces antioxidative responses and whether different plant species share a common defense mechanism or not, are not yet fully understood. Thus, the knowledge of how plants cope with Cu^{2+}-effected oxidative stress is of considerable importance in understanding the antioxidative enzyme activity changes evolved in plants.

Mentha piperita Lin. (Lamiaceae) that has been handled within this research is not only a common constituent of particularly the Indian and Eastern Asia diet, but also in the four corners of the world. It is used with other spices to give the food a special flavor and fragrance. It is also recognized for its carminative, stimulant, antispasmodic, antimutagenic, antiseptic, anti-inflammatory, antibacterial and anti-fungal activities (RUIZ DEL CASTILLO et al., 2004; DUARTE et al., 2005; FONSEKA-KRUEL and FERNANDES, 2003). Hence, it is important to know about *M. piperita*, how much it impacts from industrial pollution, which is one of today's major problems.

Though many reports concerning Cu^{2+}-excess induced oxidative stress and antioxidant responses are available (HALLIWELL and GUTTERIDGE, 1984; VAN ASSCHE and CLIJSTERS, 1990; MARSCHNER, 1995; TEWARI, 2007), there is little information available on induction of ROS generation and antioxidant responses in Cu^{2+}-deficient *Mentha piperita* leaves at different leaf positions. In the present study, the variations of antioxidant enzyme activities, chlorophyll, carotenoid and LPO levels were investigated depending on the leaf positions of *M. piperite* grown medium in Cu^{2+}-deficiency conditions and the results were compared with control and excess conditions. Antioxidant response properties were also investigated at the leaf position 6, which provided the best response to oxidative stress. The response to oxidative stress was observed to depend on the incubation time at decreasing concentrations of Cu^{2+}.

*Corresponding author

Materials and methods

Plant material and growth conditions

Seeds of *Mentha piperita* L. were disinfected with 10% H_2O_2 solution for 20 min and washed thoroughly with distilled water and germinated between wet paper towels at 25 °C in the dark for 3 d. Seedlings were grown in a growth chamber (16 h light/8 h dark) providing white fluorescent light (Philips) with an irradiance of 750 µmol m^{-2} s^{-1}, day/night temperature of 25/20 °C and 65(± 5)% relative humidity. The seedlings were grown in Hoagland and Arnon solutions until the first 8 leaves leafed out (HOAGLAND and ARNON, 1953). These solutions were permanently aerated and renewed 3-4 times a week to minimize a pH shift and nutrient depletion. During the first stage of the experiment, the seedlings were transferred to a nutrient solution composed of the Hoagland solution without Cu^{2+} and 1 mM Cu^{2+} solution. The Hoagland solution without Cu^{2+} was ensured not to include any trace of copper (ICP Model-8410, Labtam, Australia). At harvest 3, 7, 12, 14, 17 d-old leaves were weighed and used for the preparation of extracts for enzyme analysis. As a second stage of experiments, time dependent variations were also investigated in leaves of *M. piperita* grown in Cu^{2+} concentrations decreasing from 3.2 x 10^{-2} to 0 µM.

Preparation of extracts

Extracts of *M. piperita* leaves were prepared for enzyme determinations. One gram of leaf material (without the main midribs) homogenized in 4 ml 20 mM phosphate buffer (pH 7.4) was containing 50 mM β-mercaptoethanol. The homogenate was filtered and then centrifuged at 15.000 x g for 15 min. The supernatant was used for enzyme analysis. All operations (until the enzyme determination) were made out at 0 to 4 °C. β-Mercaptoethanol was not included in the homogenization buffer system for determinations of Gua-dep POD activity and LPO levels.

Determination of chlorophyll (a + b) and carotenoids concentrations

Concentrations of chlorophyll (a + b) and carotenoids were measured as described by Lichtenthaler and Wellburn (1983) after extraction with 80% acetone. The absorbance of pigment extract was measured at wavelengths 470, 646 and 663 nm. The content of Chl a, Chl b and carotenoids were estimated according to experimental equations as described in LICHTENTHALER and WELLBURN (1983).

Determination of lipid peroxidation

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 (BUEGE and AUST, 1978).

Determination of protein content

The protein content was determined by the method of Bradford using bovine serum albumin (BSA) as a standard (BRADFORD, 1976).

Assay of SOD activity

Superoxide dismutase (EC 1.15.1.1) activity was determined by the Crosti method as specific activity (CROSTI et al., 1987). One IU of enzyme activity is defined as 50% inhibition of 6-hydroxydopamine (6-OHDA) auto-oxidation under assay conditions.

Assay of CAT activity

Catalase (EC 1.11.1.6) activity was assayed in a reaction mixture containing 10.5 mM H_2O_2 in 25 mM phosphate buffer (pH 7.0). The decomposition of H_2O_2 was followed at 240 nm (ε = 39.4 mM^{-1} cm^{-1}) (AEBI, 1983). One IU of the enzyme activity was accepted as the amount of the enzyme, which decomposes 1 µmol H_2O_2 per min 25 °C.

Assay of AsA-dep POD activity

The activity of ascorbate-dependent peroxidase (EC 1.11.1.11) was measured according to NAKANO and ASADA (1981) by monitoring the rate of ascorbate oxidation at 290 nm (ε = 2.8 mM^{-1} 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. Without AsA in the test medium no change was found in absorption. For AsA-dep POD, 1 IU represents the amount of enzyme catalyzing the conversion of 1 µmol of substrate per minute.

Assay of Gua-dep POD activity

For the measurement of 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 (ε = 26.6 mM^{-1} cm^{-1}) (NAKANO and ASADA, 1981). For Gua-dep POD, 1 IU represents the amount of enzyme catalyzing the conversion of 1 µmol of substrate per minute.

Statistical analysis

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

Results

Antioxidant enzyme activities, chlorophyll/carotenoid contents and LPO levels under Cu^{2+} stress

Chlorophyll/carotenoid content and SOD, CAT, PODs enzyme activity and LPO level variations were investigated in different leaf positions of *M. piperita* (Fig. 1) grown in the conditions of 0 µM Cu^{2+} (absence), 3.2 x 10^{-2} µM Cu^{2+} (control) and 1 mM Cu^{2+} (excess) on the 14th day (Fig. 1, 3).

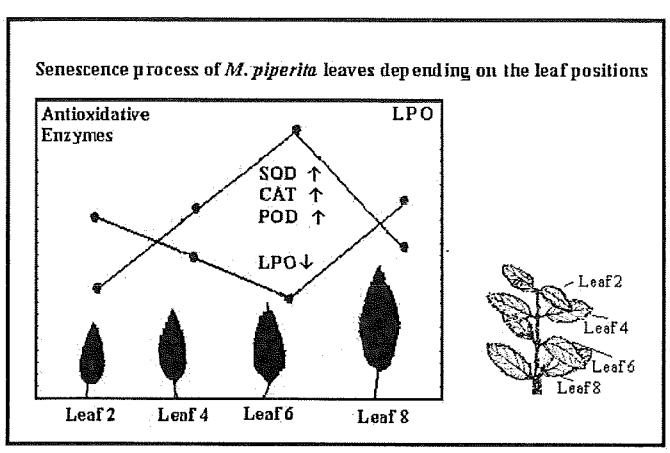

Fig. 1: Leaf positions of *M. piperita* on the stem and relationships between antioxidant enzyme activities and LPO levels (summary of Fig. 3)

Concentration of chlorophyll (Fig. 2A) and carotenoid (Fig. 2 B) and the ratio of chlorophylls to carotenoids (Fig. 2 C) in leaves were significantly lower than those of the control along the stem under absence and excess of Cu^{2+} and the maximum decreases were obtained under excess condition. As can be seen from Fig. 2, chlorophyll and carotenoid levels reached the maximum at leaf position 4 for control and leaf 4-6 showing the similar values for excess conditions ($p>0.01$) and then decreased at the following positions ($p<0.01$).

SOD activities in both absence and excess conditions of Cu^{2+} were significantly higher than those of control showing maximum increases under excess conditions ($p<0.01$) (Fig. 3 A). CAT, PODs activities of *M. piperita* leaves grown in the absence condition were higher, whereas they were lower in the presence of 1 mM Cu^{2+} solution than those of control (Fig. 3 B, C, D).

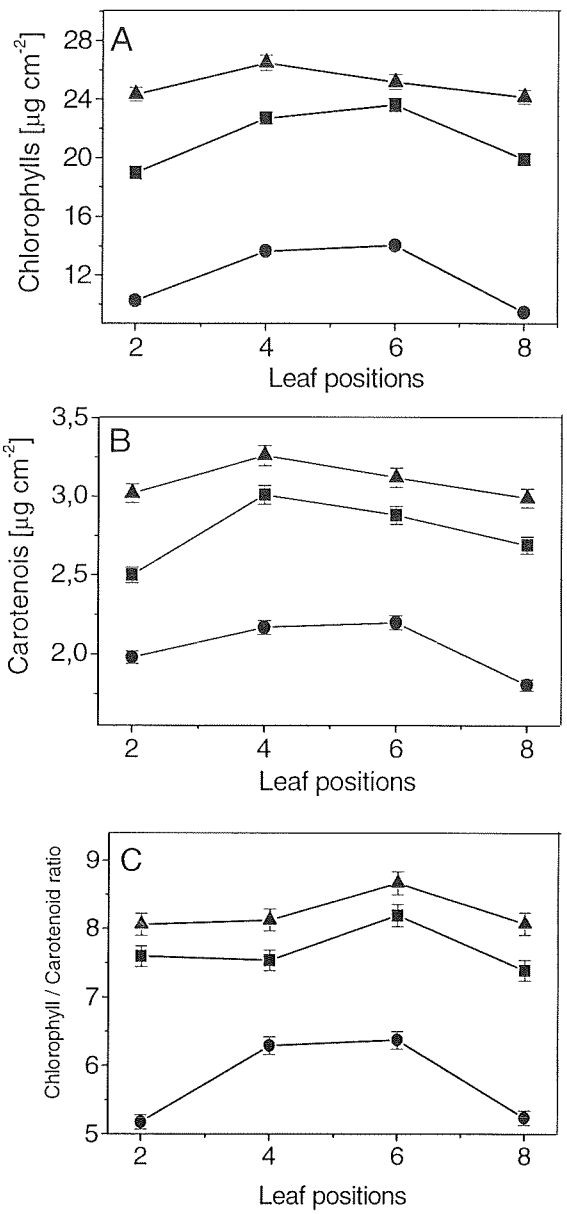

Fig. 2: Variations of total chlorophyll (A), total carotenoid (B) and the ratio of chlorophyll to carotenoid (C) , depending on the leaf positions of *M. piperita* on the 14th day (-▲-) control, 3.2 x 10^{-2} µM Cu^{2+}; (-●-), 1 mM Cu^{2+} as excess conditions; (-■-), absence of Cu^{2+} as deficiency conditions. Each data point represents the mean of three replicates.

CAT and AsA-dep POD activities increased slightly up to leaf 4 and these enzymes together with SOD levels reached the maximum levels at leaf 6 for all Cu^{2+} concentrations and they decreased as the leaves aged further ($p<0.05$). Under the absence conditions of Cu^{2+}, the activity levels in leaf position 6 are approximately 2-fold higher for SOD and AsA-dep POD, and 3-fold higher for CAT than control. However, Gua-dep POD activity in *M. piperita* leaves did not change significantly along the stems in all the conditions. It can be seen from Fig. 3 E, LPO levels in both stress conditions were significantly higher than those of control and the maximum increases were determined under excess of Cu^{2+}. LPO levels in *M. piperita* leaves along the stem showed a negative correlation with antioxidant enzyme activity variations and reached the minimum at leaf 6 ($r=-0.450$, $p<0.01$). The minimum LPO levels at leaf position 6 were determined as 8.07±0.32; 9.11±0.16 and 4.3±0.18 nmol MDA/g at 0; 1.0 mM and 3.2 x 10^{-2} µM Cu^{2+}, respectively.

Because the highest antioxidant enzyme activities and the minimum LPO levels were obtained in leaf 6, this position has been used in further experimental stages.

Antioxidant enzyme activity and LPO level variations under deficiency conditions depend on incubation period

SOD activity variations were investigated in leaf position 6 of *M. piperita* grown in a decreasing Cu^{2+} concentration from 3.2 x 10^{-2} µM (control) to 0 µM Cu^{2+} with respect to incubation period. SOD activities increased sharply with a negative correlation with Cu^{2+} concentration after 7th day and reached the maximum on the 12th day and then decreased significantly ($r=-0.687$, $p<0.01$) (Fig. 4 A).

As can be seen from Fig. 4 B, the maximum activity variations depending on the decreasing concentrations of Cu^{2+} from 3.2 x 10^{-2} µM to 0 µM were increased significantly from 48.0±1.33 to 115.08 ± 1.02 IU/mg on the 12th day ($r=-0.478$, $p<0.05$).

CAT activities remained similar under Cu^{2+} deficiency conditions up to the 7th day and then increased sharply on the 12th day with a negative correlation with decreasing Cu^{2+} concentration ($r=-0.434$, $p<0.05$) (Fig. 5 A). The maximum activity depending on the decreasing Cu^{2+} concentrations from 3.2 x 10^{-2} µM to 0 µM were increased significantly from 12.80±1.02 to 55.03±0.97 IU/mg on the 12th day. Afterwards, the activities decreased significantly below control level ($p<0.01$) (Fig. 5 B).

AsA-dep POD activities in Cu^{2+} deficient conditions of *M. piperita* reached the top figures on the 12th day showing a negative correlation ($r=-0.412$, $p<0.01$) (Fig. 6 A). AsA-dep POD activities in *M. piperita leaves* increased with a negative correlation of Cu^{2+} concentration from 5.80±0.06 (as control) to 12.05±0.34 IU/mg on the 12th day ($r=-0.412$, $p<0.01$) (Fig. 6 A, B). The activities generally did not change significantly between 17th and 23rd days of incubation for each concentration.

Gua-dep POD activities under Cu^{2+} deficiency conditions remained similar up to the 7th day and then increased sharply on the 12th day by showing a negative correlation with decreasing Cu^{2+} concentration ($r=-0.478$; $p<0.01$) (Fig. 7). These activities decreased significantly after 12th day of incubation.

A sharp acceleration in the antioxidant enzymes activities were determined with decreasing concentrations of Cu^{2+} after 3.2 x 10^{-3} µM for SOD, CAT and 6.4 x 10^{-3} µM Cu^{2+} for AsA-dep, Gua-dep PODs, respectively.

The results showed that SOD, CAT and Gua-dep POD activities changed strongly between 7-16 days.

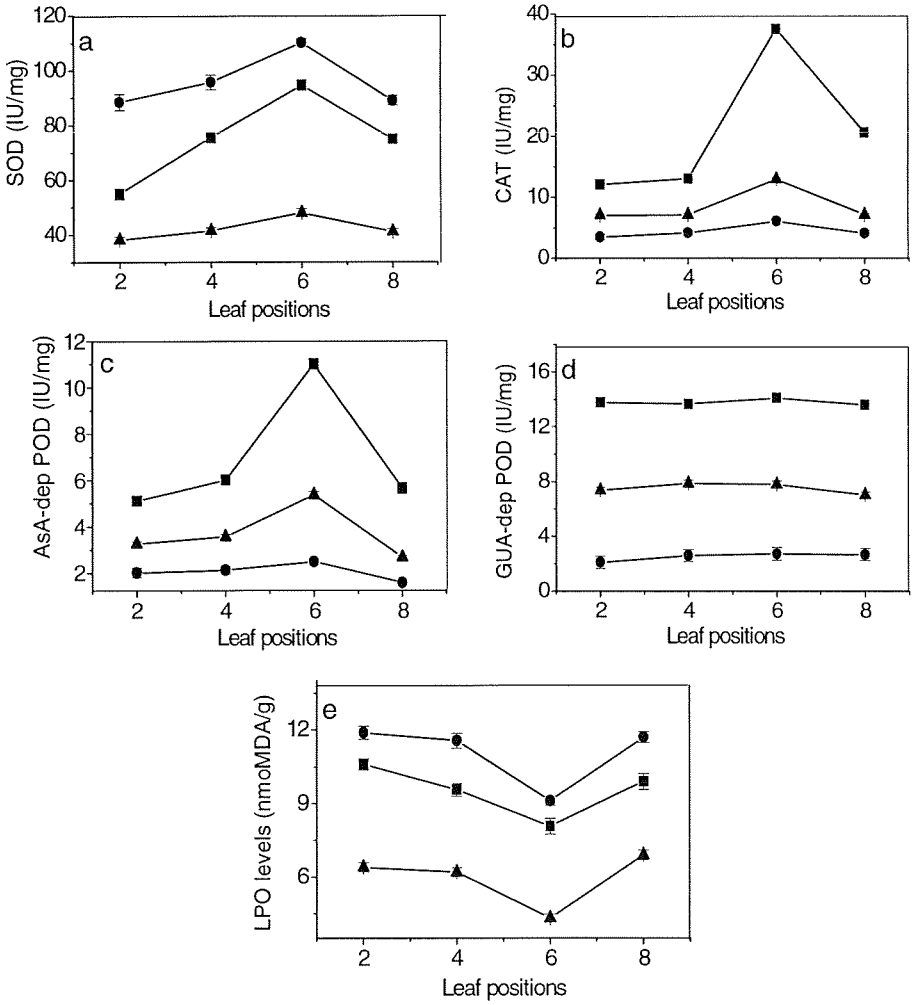

Fig. 3: Variations of superoxide dismutase (SOD) (A), catalase (CAT) (B), ascorbate-dependent peroxidase (AsA-dep POD) (C) and guaiacol-dependent peroxidase (Gua-dep POD) activity (D), depending on the leaf positions of *M. piperita* on the 14th day (-▲-) control, 3.2 x 10^{-2} µM Cu^{2+}; (-●-), 1 mM Cu^{2+} as excess conditions; (-■-), absence of Cu^{2+} as deficiency conditions. Each data point represents the mean of three replicates.

As can be seen from Fig. 8 A, the LPO level variations in *M. piperita* leaves showed a negative correlation with decreasing Cu^{2+} concentrations over the treatment period ($r=-0.450$, $p<0.01$). The correlation value at 23rd day ($r=-0.970$, $p<0.01$) were significantly higher than that of the 3rd day ($r=0.496$, $p<0.01$) (Fig. 8 B). LPO levels remained similar up to 7th day and then showed a minimum at 12th day. LPO levels were increased in the following incubation period.

Discussion

Cu^{2+} is a structural and catalytic component of many proteins involved in a variety of metabolic pathways and it is responsible for many alterations of the plant cell (De Vos et al., 1989; Liu and Xiong, 2005; Liu et al., 2004; Maksymiec, 1997). Hence, either deficient or in excess conditions, Cu^{2+} can cause disorders in plant growth and development by adversely affecting important physiological process in plants. In particular photosynthetic electron transport is altered under both Cu^{2+} deficiency and excess conditions. This is also evident from our study, where Cu^{2+} deficiency and especially excess conditions resulted in a significant loss in the chlorophyll and carotenoid contents of *M. piperita* leaves when compared with control. Copper-deficiency in *M. piperita* might cause chloroplasts ultra-structure and electron transport system damage via decreasing pigment contents and increasing ROS scavenging enzyme activities.

The decreasing of chlorophyll content could be due to peroxidation of chloroplasts membranes (thylakoids) mediated by Cu^{2+} as suggested earlier in excess conditions (Baszynski et al., 1988; Sandmann and Böger, 1980; Nyitrai et al., 2003). Another reason for reduction in chlorophylls concentrations in *M. piperita* might be probably caused by interaction of Cu^{2+} to -SH groups of enzymes of chlorophyll biosynthesis.

Decreased carotenoids content due to both deficiency and excess conditions when compared with control might be protective functions of carotenoids, known to be potent quenchers of ROS, particularly singlet oxygen. Also the chlorophyll and carotenoid contents and the ratio of chlorophylls to carotenoids were lower than the control values in all leaf positions. Lower values for the ratio (a+b) / (x+c) were an indicator of stress and damage to the photosynthetic apparatus which was expressed by a faster breakdown of chlorophylls than carotenoids (Lichtenthaler, 1987; Lichtenthaler, 1993).

The protective mechanisms adapted by plants to scavenge free radicals and peroxides include several antioxidant enzymes (Allen, 1995; Mittler et al., 2004). Overexpression of genes encoding these

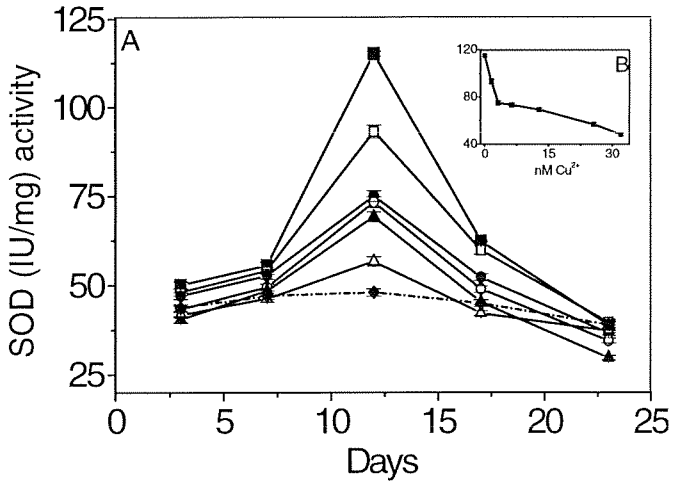

Fig. 4: Superoxide dismutase (SOD) activity variations (A) in leaf 6 of *M. piperita* in Cu^{2+} deficiency conditions depending on time: (-■-) 0 µM, (-□-) 1.6 x10^{-3} µM, (-●-) 3.2 x 10^{-3} µM, (-○-) 6.4 x 10^{-3} µM, (-▲-) 1.28 x 10^{-2} µM, (-△-) 2.56 x 10^{-2} µM and (-◆-) 3.2 x 10^{-2} µM Cu^{2+} (B) SOD activities depending on Cu^{2+} concentrations on 12^{th} day. Each data point represents the mean of three replicates.

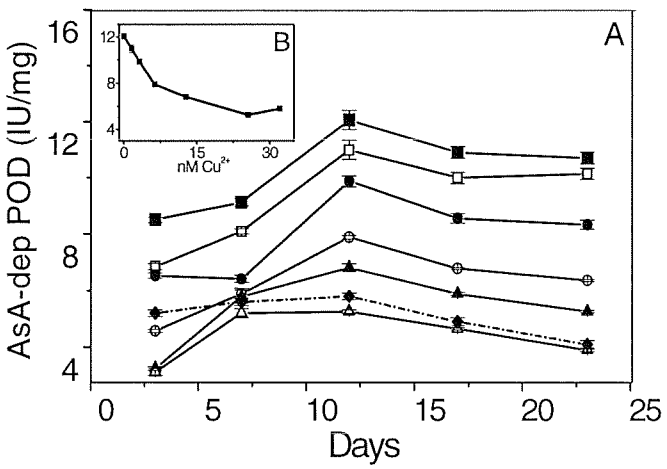

Fig. 6: Ascorbate-dependent peroxidase (AsA-dep POD) activity variations (A) in leaf 6 of *M. piperita* in Cu^{2+} deficiency conditions depending on time: (-■-) 0 µM, (-□-) 1.6 x 10^{-3} µM, (-●-) 3.2 x 10^{-3} µM, (-○-) 6.4 x 10^{-3} µM, (-▲-) 1.28 x 10^{-2} µM, (-△-) 2.56 x 10^{-2} µM and (-◆-) 3.2 x 10^{-2} µM Cu^{2+} (B) AsA-dep POD activities depending on Cu^{2+} concentrations on 12^{th} day. Each data point represents the mean of three replicates.

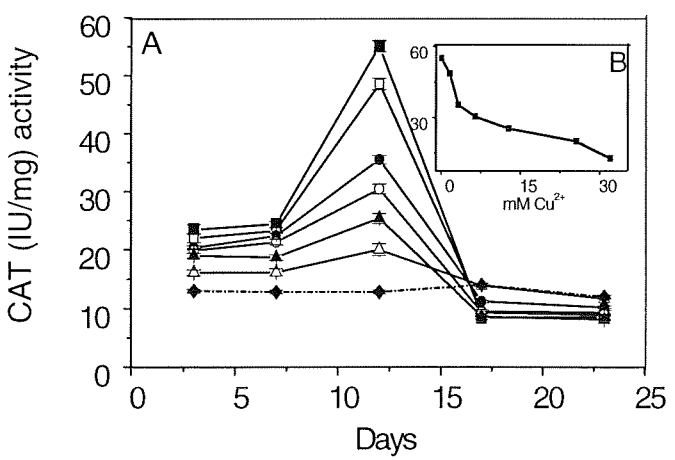

Fig. 5: Catalase (CAT) activity variations (A) in leaf 6 of *M. piperita* in Cu^{2+} deficiency conditions depending on time: (-■-) 0 µM, (-□-) 1.6 x 10^{-3} µM, (-●-) 3.2 x 10^{-3} µM, (-○-) 6.4 x 10^{-3} µM, (-▲-) 1.28 x 10^{-2} µM, (-△-) 2.56 x 10^{-2} µM and (-◆-) 3.2 x 10^{-2} µM Cu^{2+} (B) CAT activities depending on Cu^{2+} concentrations on 12^{th} day. Each data point represents the mean of three replicates.

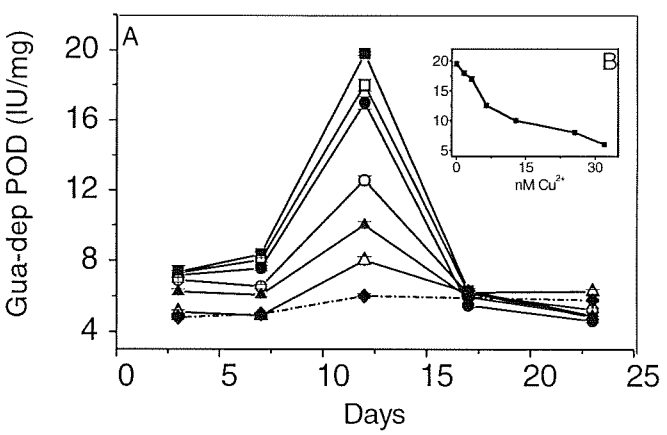

Fig. 7: Guaiacol-dependent peroxidase (Gua-dep POD) activity variations in leaf 6 of *M. piperita* in Cu^{2+} deficiency conditions depending on time: (-■-) 0 µM, (-□-) 1.6 x 10^{-3} µM, (-●-) 3.2 x 10^{-3} µM, (-○-) 6.4 x 10^{-3} µM, (-▲-) 1.28 x 10^{-2} µM, (-△-) 2.56 x 10^{-2} µM and (-◆-) 3.2 x 10^{-2} µM Cu^{2+} (B) Gua-dep POD activities depending on Cu^{2+} concentrations on 12^{th} day. Each data point represents the mean of three replicates.

enzymes in several transgenic plant species conferring protection against free radicals has been demonstrated (FOYER et al., 1994). However, the response of antioxidant enzymes to copper, and in general to metals, remains controversial and can vary among plant species (LOMBARDI and SEBASTIANI, 2005). Many reports show a copper-dependent increase in the activity SOD, CAT and PODs (WECKX and CLIJSTERS, 1996; VAN ASSCHE and CLIJSTERS, 1990; RAZINGER et al., 2007) while, in contrast, others reports found that SOD and CAT can be inhibited by excess copper (LUNA et al., 1994; PALMA et al., 1987). In our study, the activity of SOD in *M. piperita* leaves increased both deficiency and also excess conditions and SOD showed a positive correlation with CAT, AsA-dep and Gua-dep POD along the stems under absence stress conditions (r=0.950; r=0.890; r=0.855, $p<0.01$) when compared with control. These results were also observed under excess condition, whereas SOD changed negatively with all these enzymes under excess Cu^{2+} conditions (r=-0.938; r=-0.614; r=-0.942, $p<0.01$). SOD, CAT, PODs display a co-operative function for preventing a partial protection against oxidative stress under absence of Cu^{2+} in grown medium of *Mentha piperita*. This situation caused lower LPO levels in the absence of Cu^{2+} than excess conditions, while higher LPO levels than control. These results showed that reduced ETS functioning is likely to result in a spillover of electrons from ETS components of chloroplasts and mitochonria to O_2 and generate $O_2^{\cdot-}$. Copper-deficiency, apart from increasing $O_2^{\cdot-}$ production, also increased the activity of SOD, suggesting induction of oxidative stress in *M. piperita*

In the current study, there might be some possibilities for the decreased CAT and PODs activities under excess conditions. One was Cu^{2+} that bound or replaced some components such as Fe^{2+} in the enzyme (DE VOS et al., 1989; DAS et al., 1978). Also, it was possible

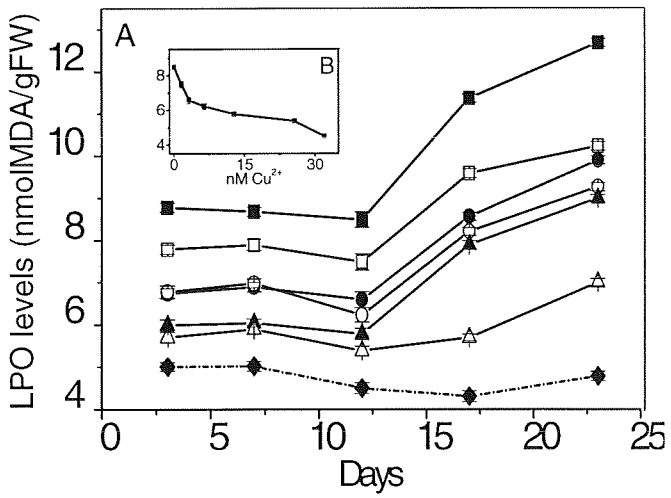

Fig. 8: The variations of lipid peroxidation (LPO) levels (A) in leaf 6 of *M. piperita* in Cu^{2+} deficiency conditions depending on time: (-■-) 0 µM, (-□-) 1.6 x 10^{-3} µM, (-●-) 3.2 x 10^{-3} µM, (-○-) 6.4 x 10^{-3} µM, (-▲-) 1.28 x 10^{-2} µM, (-△-) 2.56 x 10^{-2} µM and (-◆-) 3.2 x 10^{-2} µM Cu^{2+} (B) LPO levels depending on Cu^{2+} concentrations on 12th day. Each data point represents the mean of three replicates.

that CAT and PODs in *M. piperita* would be more sensitive to excess Cu^{2+} since it readily bound to thiol groups and thereby inactivated the thiol-containing enzyme. Another reason for the decrease in these enzyme activities might seem to be related with the redox active nature of Cu^{2+}, which catalyses the formation of extremely reactive hydroxyl radicals and these radicals cause the inhibition of enzyme synthesis such as CAT and POD (PINTO et al., 2003).

Another important finding of our study is that the increase in SOD, CAT and AsA-dep POD activities ($r=0.483$; $r=0.501$; $r=0.511$ $p<0.01$) and decrease in LPO levels ($r=-0.469$, $p<0.01$) depending on the leaf positions from 2 to 6, may be evidence of increased antioxidant defense system against Cu^{2+} deficiency. These results are also coherence with excess condition. On the other hand, the decrease in SOD, CAT, AsA-dep POD activities ($r=-0.425$; $r=-0.368$; $r=-0.435$, $p<0.01$) and increase in LPO levels ($r=0.424$, $p<0.01$) were observed depending on the leaf positions from leaf 6 to 8. These might be explained by the fact that the senescence process of *M. piperita* leaves suppressed the antioxidant defense system due to diminished activities of these enzymes might cause exposure of tissues and cells to more peroxidative attacks.

The antioxidant defense system of *M. piperita* leaves (leaf 6) showed different responses depend on the decreasing Cu^{2+} concentration and incubation time. In spite of increases in SOD and AsA-dep POD activities ($r=0.526$ and $r=0.448$, $p<0.01$), LPO levels remain their levels above the control levels during first 7 days. However, increases in SOD, CAT, AsA-dep and Gua-dep POD enzyme activities compared with control ($r=0.742$; $r=0.544$; $r=0,429$ and $r=0.694$, $p<0.01$) between 7th and 12th day provide a temporary protection due to partial decreasing of LPO levels ($r=-0.404$, $p<0.01$). All the investigated antioxidant enzyme activities peaked at 12th day and decreased immediately between 16th and 22nd days in all Cu^{2+} deficiency conditions. The increase in the antioxidant enzyme activities accelerated approximately 3-fold for SOD and CAT with the decreasing Cu^{2+} concentrations from 3.2 x 10^{-3} µM and 2-fold for AsA and Gua-dep PODs from 6.4 x 10^{-3} µM Cu^{2+} as compared to the higher Cu^{2+} concentrations at 12th day. This situation exhibited similar trend for LPO variations in leaves which showed a negative correlation with Cu^{2+} concentration. These results show that the prevention of membrane damage of *M. piperita* leaves could be achieved of collaboration of all these antioxidant enzymes activities. This situation might be explained by a serious imbalance between production of ROS and antioxidant defense systems (HALLIWELL and GUTTERIDGE, 2001).

After 12th day; SOD, CAT, PODs activities ($r=-0.735$; $r=-0.762$; $r=-0.436$; $r=-0.729$, $p<0.01$) decreased, whereas LPO levels ($r=0.420$, $p<0.01$) increased significantly depend on time. So that the correlation values between LPO levels and decreasing Cu^{2+} concentration were significantly higher on the 23rd than 12th day. This situation caused that decreases in all investigated antioxidant enzyme activities owing to time and decreasing of Cu^{2+} concentration caused acceleration of senescence process of *M. piperita* leaves after the 12th day.

Despite the higher activities of SOD, CAT, AsA and Gua-dep PODs compared to control in both research conditions, all the leaf positions along the stem in absence of Cu^{2+} and leaf position 6 in Cu^{2+}-deficiency conditions depend on the incubation period, increases in LPO levels were also determined. These results lead to the conclusion that *Mentha piperita* responded against oxidative stress under Cu^{2+} deficiency conditions. However, this defense capacity may not be sufficient to prevent membrane peroxidation, which is an indicator of oxidative damage. At low copper concentrations, the oxidative stress response of SOD and CAT was observed to be 3 times faster at copper concentrations of 3.2 x 10^{-3} µM or lower at the 12th day and similarly for AsA and Gua-dep POD; it was twice faster at copper concentrations of 6.4 x 10^{-3} µM or lower. It was observed that this situation happened again for LPO levels with decreasing concentrations of Cu^{2+} at 12th day. However, these sharp increases in all the antioxidant enzyme activity responses were not enough to prevent oxidative stress conditions.

References

AEBI, H.E., 1983: Catalase, in methods of enzymatic analysis. In: Bergmeyer, H.U. (ed.), Vol. 3, Verlag Chemie: Deerfield Beach FL.

ALLEN, R.D., 1995: Dissection of oxidative stress tolerance using transgenic plants. Plant Physiol. 107, 1049-1054.

ASADA, K., 1994: Official methods of analysis. In: Foyer, C., Mullineaux, P. (eds.), Causes of photooxidative stress and amelioration of defense systems in plants, CRC Press, Boca Raton.

BASZYNSKI, T., TUKENDORF, M., RUSZKOWSKA, M., SKORZYNSKA, E., MAKSYMIEC, W., 1988: Characteristics of the photosynthetic apparatus of copper. J. Plant Physiol. 132, 703-713.

BRADFORD, M.M., 1976: A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

BUEGE, J.A., AUST, S.D., 1978: Microsomal lipid peroxidation. Methods Enzymol. 52, 464-478.

CROSTI, N., SERVIDEN, T., BAJER, J., SERRA, A., 1987: Modification of 6-hydroxydopamine technique for the correct determination of superoxide dismutase. J. Clin. Chem. Clin. Biochem. 25, 265-272.

DAS, P.K., KAR, M., MISHRA, D., 1978: Nickel nutrition of plants: Effects of nickel on some oxidize activities during rice seed germination. Z. Pflanzenphysiol. 90, 225-233.

DE VOS, C.H.R., SCHAT, H., VOOIJS, R., ERNST, W.H.O., 1989: Copper induced damage to the permeability barrier in root of *Silene cucubalus*. J. Plant Physiol. 135, 164-169.

DUARTE, M.C., FIGUEIRA, G.M., SARTORATTO, A., REHDER, V.L., DELARMELINA, C., 2005: Anti-*Candida* activity of Brazilian medicinal plants. J. Ethnopharmacol. 97, 305-311,

FONSEKA-KRUEL, V.S., FERNANDES, P.V., 2003: Coleção de Plantas

Medicinais, Wrst ed. Instituto de Pesquisas Jardim Botânico do, Rio de Janeiro.

FOYER, C.H., DESCOURVIERES, P., KUNERT, K.J., 1994: Protection against oxygen radicals: an important defense mechanism studied in transgenic plants. Plant Cell Environ. 17, 507-523.

HALLIWELL, B., GUTTERIDGE, J.M.C., 1984: Oxygen toxicity, oxygen radicals transition metals and disease. Biochem. J. 219, 1-14.

HALLIWELL, B., GUTTERIDGE, J.M.C., 2001: Free radicals in biology and medicine. Oxford University Press, 261-271.

HOAGLAND, D.R., ARNON, D.I., 1953: The water culture method for growing plants without soil. California Agric. Exp. Stat. Univ. Calif, Berkeley Circle.

LICHTENTHALER, H.K., 1987: Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods Enzymol. 48, 350-382.

LICHTENTHALER, H.K., 1993: The plant prenyllipids, including carotenoids, chlorophylls and prenylquinones. In: Moore, T.S. (ed.), Lipid metabolism in plants, 427-470. CRC Press Inc., Boca Raton.

LICHTENTHALER, H.K., WELLBURN, A.R., 1983: Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. Biochem. Soc. Trans. 11, 591-592.

LIN, C.C., KAO, C.H., 2000: Effect of NaCl stress on H_2O_2 metabolism in rice leaves. Plant Growth Regul. 30, 151-155.

LIU, J., XIONG, Z.T., 2005: Differences in accumulation and physiological response to copper stress in three populations of *Elsholtzia haichowensis*. Water Air Soil Poll. 168, 5-16.

LIU, J., XIONG, Z.T., LI, T., HUANG, H., 2004: Bioaccumulation and ecophysiological responses to copper stress in two populations of *Rumex dentatus* L. from Cu contaminated and non-contaminated sites. Environ. Exp. Bot. 52, 43-51.

LOMBARDI, L., SEBASTIANI, L., 2005: Copper toxicity in *Prunus cerasifera*: growthand antioxidant enzymes responses of in vitro grown plants. Plant Sci. 168, 797-802.

LUNA, C.M., GONZALES, C.A., TRIPPI, V.S., 1994: Oxidative damage caused by an excess of copper in oat leaves. Plant Cell Physiol. 35, 11-15.

MARSCHNER, H., 1995: Mineral nutrition of higher plants, second ed. Academic Press, London, 337-347.

MAKSYMIEC, W., 1997: Effect of copper on cellular process in higher plants. Photosynthesis 34, 321-342.

MITTLER, R., VANDERAUWERA, S., GOLLERY, M., BREUSEGEM, F.V., 2004: Abiotic stress series. Reactive oxygen genes network of plants. Trends Plant Sci. 9, 490-498.

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

NYITRAI, P., BOKA, K., GASPAR, L., SARVARI, E., LENTI, K., KERESZTES, A., 2003: Characterization of the stimulating effect of low-dose stressors in maize and bean seedlings. J. Plant Physiol. 160, 1175-1184.

PALMA, J.M., GOMEZ, M., YANEZ, J., DEL RIO, L.A., 1987: Increased levels of peroxisomal active oxygen-related enzymes in copper-tolerant pea plants. Plant Physiol. 85, 570-574.

PINTO, E., SIGAUD-KUTNER, T.C.S., LEITAO, M.A.S., OKAMOTO, O.K., MORSE, D., COLEPICOLO, P., 2003: Heavy metal-induced oxidative stress in algae. J. Plant Physiol. 39, 1008-1018.

RAZINGER, J., DERMASTIA, M., DRINOVEC, L., DROBNE, D., ZRIMEC, A., 2007: Antioxidative responses of duckweed (*Lemna minor* L.) to short-term copper exposure. Environ. Sci. Pollut. R. 14, 194-201.

RUIZ DEL CASTILLO, M.L., BLANCH, G.P., HERRAIZ, M., 2004: Natural variability of the enantiomeric composition of bioactive chiral terpenes in *Mentha piperita*. J. Chromatogr. 29, 87-93.

SALISBURY, F.B., ROSS, C.W., 1992: *Plant Physiology*, 4th edn. Wadsworth, Belmont, C. A.

SANDMANN, G., BOGER, P., 1980: Copper-mediated lipid peroxidation processes in photosynthetic membranes. Plant Physiol. 66, 797-800.

STIBOROVÁ, M., DITRICHOVÁ, M., BREZINOVÁ, A., 1988: Mechanism of action of Cu^{2+}, Co^{2+} and Zn^{2+} on ribulose,1-5-bisphosphate carboxylase from barley (*Hordeum vulgare* L.). Photosynthetica 22, 161-167.

TEWARI, R.K., KUMAR, P., SHARMA, P.N., 2006: Antioxidant responses to enhanced of superoxide anion radical and hydrogen peroxide in the copper-stressed mulberry plants. Planta 223, 1145-1153.

VAN ASSCHE, F.F., CLIJSTERS, H., 1990: Effects of metals on enzyme-activity in plants. Plant Cell Environ. 13, 95-206.

VIARENGO, A., 1985: Biochemical effects of trace metals. Mar. Pollut. Bull. 16, 153-158.

WECKX, J.E.J., CLIJSTERS, H.M.M., 1996: Oxidative damage and defense mechanisims in primary leaves of *Phaseolus vulgaris* as a result of root assimilation oftoxic amounts of copper. Plant Physiol. 96, 506-512.

YRUELA, I., 2005: Copper in plants. Braz. J. Plant Physiol. 17, 145-156.

ZHANG, J., KIRKHAM, M.B., 1996: Enzymatic responses of the ascorbate-glutathione cycle to drought in sorghum and sunflower plants. Plant Sci. 113, 139-147.

Address of the author:
L. Tarhan, Tel.: +90 232 420 48 82-1317; Fax: +90 232 420 48 95; E-mail: leman.tarhan@deu.edu.tr