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ROKA (*ERUCA SATİVA*) BİTKİSİNİN KÖK VE GÖVDESİNDEKİ ANTİOKSİDAN ENZİM AKTİVİTELERİ, METAL BİRİKİMİ, BAZI FİZYOLOJİK VE BÜYÜME PARAMETRELERİNDEKİ DEĞİŞİMLERİN ARAŞTIRILMASI

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Özet. Bu çalışmada, farklı Mn^{+2} (10 - 5000 μM) veya Fe^{+2} (50 – 5000 μM) konsantrasyonlarının varlığında *Eruca sativa* bitkisindeki büyüme geriliği ile ilişkili olarak köklerde ve gövdede antioksidatif cevabın karşılaştırılmasını amaçladık. Aşırı Mn^{+2} veya Fe^{+2} bitkilerin büyümesini anlamlı olarak inhibe etti. Her iki dokuda da, Mn^{+2} ve Fe^{+2} stresinde MDA, prolin ve H_2O_2 seviyelerinde, elektrolitik iletkenlik, SOD ve GPX aktivitelerinde anlamlı artış izlendi. *Eruca sativa* bitkisinde, dışarıdan artan konsantrasyonlarda uygulanan Mn^{+2} ve Fe^{+2}, bitkinin köklerinde ve gövdesinde uygulanan metalin aşırı birikimine sebep oldu. Aşırı Mn^{+2} köklerde ve gövdede Fe^{+2} ve Zn^{+2} alınımını anlamlı olarak azaltırken Cu içeriğini arttırdı. Sonuç olarak, yüksek Mn^{+2} veya Fe^{+2} içeriği oksidatif stresi arttırarak büyümenin baskılanmasından sorumlu tutulabilir.

Anahtar Kelimeler: Roka (*Eruca sativa*) bitkisi, Mn^{+2} ve Fe^{+2} stresi, metal alımı, lipid peroksidasyonu, H_2O_2 içeriği, proline içeriği.

STUDY OF THE CHANGE IN ANTIOXIDATIVE ENZYME ACTIVITIES, METAL ACCUMULATION, SOME PHYSIOLOGICAL AND GROWTH PARAMETERS OF SHOOT AND ROOT IN ROCA (*ERUCA SATIVA*) PLANT

Abstract: In this study, we aimed to compare some antioxidative responses, which is associated with the growth reduction, of shoots and roots of *Eruca sativa* plant in the presence of different concentration of Mn^{+2} (10 - 5000 μM) or Fe^{+2} (50 – 5000 μM). The excess of Mn^{+2} or Fe^{+2} caused a significant inhibition on the plant growth. Significant increases in MDA, SOD, GPX, proline, electrolytic leakage and H_2O_2 levels were observed in both shoots and roots stressed under Mn^{+2} and Fe^{+2}. Mn^{+2} treatment caused a greater decrease in growth than Fe^{+2} treatment in both tissues. The contents of total chlorophyll and carotenoid in the leaves of the plant were also reduced by the increasing the Mn^{+2} or Fe^{+2} concentrations. Cu uptake was increased in the presence of excess Mn^{+2} in both tissues, although Fe^{+2} and Zn^{+2} contents were significantly reduced. Consequently, it can be concluded, that the high Mn^{+2} or Fe^{+2} contents in growth media can be the responsible for the growth inhibition by enhancing the oxidative stress.

Key words: Roca (*Eruca sativa*) plant, Mn^{+2} and Fe^{+2} stresses, metal uptake, lipid peroxidation, H_2O_2 content, proline content

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1. INTRODUCTION

Phytotoxicity of heavy metals such as Mn^{+2} and Fe^{+2} is long known and well documented. However, the mechanisms of metal toxicity induction are not fully understood. The phytotoxicity of the heavy metals due to industrial pollution has serious implications in soil degradation (Goos et al., 2004). This may reduce both the quality and productivity of plants. Heavy metals differ according to their role in metabolic functions. Microelements, such as Fe^{+2} and Mn^{+2} are essential and are involved in numerous physiological processes (Nikolic and Kastori, 2000); however at high concentrations, they are strongly toxic and impair plant growth.

Studies on heavy metal tolerance in plants are important because of the limitations imposed by neutral and man-made environmental stresses for food production (Foy et al., 1988). Missing the efficiency of the use of cultivated land and increase the utilization of marginal soils are necessary to feed the growing human population. Because of the economic impact of stresses and the large amount of energy required to alter the environment to suit the plant, it is becoming increasingly important to utilize the existing technologies and to develop new ones in order to develop plants that are adapted to stress (Tal, 1983).

Metal cofactor such as Mn^{+2}, Fe^{+2}, Cu^{+2} and Zn^{+2} are essential components of many enzymes and proteins involved in various metabolic processes of living organisms. Metal ions have been known to regulate gene expression as well as protein function (Ishikawa et al., 2003). Manganese is a common metal in the Earth's crust and its presence in soils mainly results from Mn^{+2} in the parent material. Manganese uptake by plants mainly occurs in the reduced-bivalent form, thus its availability increases in acidic soils or anaerobic conditions. Plant species; differ considerably in resistance to excess manganese. First visible symptoms are brown spots on leaves followed by chlorosis, necroses and shedding of leaves. It is also an essential micronutrient for plants (Nikolic and Kastori, 2000; Goos et al., 2004).

High Mn^{+2} levels in soil may lead to plant nutrient imbalances, especially in relation to other metals such as Fe^{+2}, Zn^{+2} and Cu^{+2}. In general, nutrient uptake, especially in relation with elements entering to the roots by diffusion, may be hampered by Mn^{+2} due to Mn^{+2} inhibition of root hair production and reduction of stomata dimensions (Lidon, 2004). High substrate Mn^{+2} may thus reduce plant growth due to other nutrient deficiencies instead of Mn^{+2} toxicity (Foy et al., 1988).

Iron is an essential metal which has pivotal importance in free radical defense mechanisms of living cells. Excessive concentrations of iron and its compounds have demonstrated the potential to induce free radical production, which plays an important role in the onset of peroxidation of membrane lipids (Halliwell and Guteridge, 1985). Large amounts of Fe^{+2} in plants can give rise to the formation of oxygen radicals, which are highly phytotoxic and responsible for protein degradation and peroxidation of membrane lipids. Varieties differ in susceptibility to Fe^{+2} toxicity.

Under stress conditions, plants besides producing antioxidants also accumulate compatible solutes in the cytosol, such as proline, that originally were thought to function as osmotic buffers. However, apart from osmotic adjustment they seem to play a role in maintaining the natural state of macromolecules probably by scavenging ROS (Xiong and Zhou, 2002)

Molecular oxygen, is although essential for the existence and survival of living organisms through the formation of reactive oxygen species (ROS). The active oxygen species generated in the plant cells are

normal by production of these species such as superoxide, hydrogen peroxide, hydroxyl radicals, results in the plant exposed to stress conditions including metal stress (Xiong and Zhou, 2002). All aerobic organisms possess the means to protect themselves against toxic effects of oxygen species. Thus important factors of iron and manganese toxicity are generation of oxidative stress. Tolerance and protective mechanisms have evolved to scavenge free radicals and peroxidase generated during various metabolic reactions (Foyer et al., 1994). These protective mechanisms include antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase GPX) and antioxidant compounds. The enzymatic antioxidant system include SOD, which catalyzes the reaction from $O_2^{\bullet-}$ to H_2O_2, and CAT, APX and GPX which are able to detoxify the to H_2O_2 produced (Halliwell and Gutteridge, 1985; Foyer et al., 1994).

The aim of the present study was to investigate the effects on growth, lipid peroxidation, proline and H_2O_2 accumulation, some antioxidant enzyme activities, metal accumulation and metal uptake of shoots and roots of *Eruca sativa* in response to excess concentrations of Mn^{+2} and Fe^{+2}.

2. MATERIAL AND METHODS

Plants Material and Metal Treatment

Seeds were surface sterilized with 0.1% (w / v) sodium hypochlorite solution for 5 min. and thoroughly rinsed with distilled water and allowed to germinate in petri plates lined with filter papers (Whatman no:1). 7 days-old seedlings were transferred to containing grated half strength Hougland nutrient solution in the absence (control) and presence of varying concentrations $MnCl_2$. $4H_2O$ (10-5000 µM Mn^{+2}) and $FeSO_4.7\ H_2O$ (50-5000 µM Fe^{+2}). The composition of nutrient solution was: 1.02 g L^{-1} KNO_3, 0.492 g L^{-1} $Ca(NO_3)_2$, 0.23 g L^{-1} KH_2PO_4, 0.49 g L^{-1} $MgSO_4.7\ H_2O$, 2.86 mg L^{-1} H_3BO_3, 1.81 mg L^{-1} $MnCl_2.4\ H_2O$, 0.08 mg L^{-1} $CuSO_4.5\ H_2O$, 0.22 mg L^{-1} $ZnSO_4.7\ H_2O$, 0.09 mg L^{-1} $NaMoO_4.H_2O$ and $FeSO_4.7\ H_2O$ (0.5%) contained 0.6 mL L^{-1} (0.4%) tartaric acid. Nutrient solutions were renewed every 3 days. The pH of the medium varied from 5.8 to 6.0.Plants were grown in a controlled environment growth chamber with a 16 - h, 25 ° C day and an 8 - h, 20 ° C night regime, and 60 – 70% relative humidity. Shoot and root samples were harvested after 0, 1, 3, and 6 days of Mn^{+2} or Fe^{+2} treatment from different sets of plants for assay of various antioxidative enzymes. All of the experiments were repeated at least three times.

Metal analysis

Harvested plants (6 days of Fe^{+2} or Mn^{+2} treatment) were washed thoroughly with distilled water for analysis of metal accumulation. The shoots and roots of plants were separated manually, dried in an oven 80 ℃ for four day. The dried material was wet-ashed in a HNO_3 and $HClO_4$ mixture (3 : 1) (V / V) and the Fe^{+2}, Mn^{+2} , Cu^{+2} and Zn^{+2} concentrations were determined by atomic absorption spectrometry (Varian FS 2000).

Growth analysis

After 0 and 6 days of metal treatment, 1 g plants for each group were taken at random and divided into separate shoot and root fractions. The fresh weights of shoots and roots were weighed, and root lengths were measured. The samples were then dried in a forced draft oven at 70 ℃ for 24 h, and the dry weights (g g fw^{-1}) were determined.

Determination of Proline and H_2O_2 Content

Proline content was determined according to the modified method of Bates et al. (1973). 0.5 g of shoot and root tissues from control and Mn^{+2} or Fe^{+2} treated plants were

homogenized in 1 mL of 5 % sulfosalicylic acid solution using homogenizer. The homogenate was then centrifuged at 13 000 g for 10 min. One milliliter of the supernatant was then added into a test tube to which 1 mL of glacial acetic acid and 1 mL of freshly prepared acid ninhydrin solution were added (1.25 g ninhydrin dissolved in 30 mL of glacial acetic acid and 20 mL of 6 M orthophosphoric acid). Tubes were incubated in a water bath for 1 h at 95 º C and then allowed to cool to room temperature. Two milliliters of toluene was added and mixed on a vortex mixture for 20 s in a fume hood. The test tubes were allowed to stand for at least 10 min to allow the separation of toluene and aqueous phase. The toluene phase was carefully pipetted out into a glass test tube and the absorbance was measured at 520 nm in a spectrophotometer. The concentration of proline was calculated from a proline standard curve. The concentration of proline was expressed as μmol g fw^{-1}.

The hydrogen peroxide content was determined according to Jana and Choudhuri (1981). Aliguats of fresh shoots and roots were homogenized in 50 mM potassium phosphate, pH 6.5 and centrifuged at 10 000 g for 25 min. The solution was mixed with 1 % titanium chloride (in concentrated HCl) and then centrifuged at 10 000 g for 15 min The absorbance of the supernatant was measured at 410 nm and the H_2O_2 content calculated using 0.28 $\mu M^{-1}cm^{-1}$ as extinction coefficient.

Chlorophyll and carotenoid contents

Concentration of chlorophylls and carotenoids were determined in DMSO extract of the young fully expanded leaf by the method of Lichtenthaler and Wellburn (1983). The homogenate was centrifuged at 4000 × *g* for 10 min to remove the residue. The color intensity of clear supernatant was measured at 665, 645 and 470 nm for chlorophyll a, chlorophyll b and carotenoids, respectively. Results have been expressed as mg chlorophyll or carotenoids mg g fw^{-1}.

Electrical conductivity as a measure of ion leakage

The treated plant material (750 mg) was taken and washed with double distilled water. They were then transferred to 100 ml of deionized water and left for 24 h to facilitate maximum ion leakage. The electrical conductivity of the water as a measure of ion leakage was measured after 24 h according to Devi and Prasad, 1998.

Enzyme Extracted and Enzyme Activities Assays

Fresh shoot and root samples weighting about 1 g were homogenized using chilled mortar and pestle in 5 ml of cold 20 mM potassium phosphate buffer (pH 7.0) containing 1.0% insoluble polyvinyl pyrolidone (PVP) in ice bath. The homogenates were centrifuged at 12 000 *g* for 30 min. The supernatant was stored at 4 ºC and used for enzyme assays.

Ascorbate peroxidase (EC. 1.11.1.11) activity was measured immediately in fresh extracts and was assayed as describes by Nakano and Asada (1981) using a reaction mixture containing 25 mM potassium phosphate buffer (pH 7.0), 0.1 mM H_2O_2, 0.5 mM ascorbate and 0.1 mM EDTA. The hydrogen peroxide-dependent oxidation of as was followed by a decrease in the absorbance at 290 nm. The activity of APX was calculated using the extinction coefficient of 2.8 mM^{-1} cm^{-1}.

Guaiacol peroxidase (EC. 1.11.1.7) activity was determined in a reaction mixture containing 25 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2, 10 mM guaiacol and enzyme extracts. The increase of absorbance, due to tetra guaiacol formation, was recorded at 470 nm and enzyme activity was determined

using and extinction coefficient of 26.6 mM^{-1} cm^{-1} (Nakano and Asada, 1981).

Catalase (EC 1.11.1.6) activity was determined by monitoring the described of H_2O_2 which was carried out by measuring the decrease in absorbance at 240 nm of a reaction mixture containing 25 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2 and enzyme extract (Aebi, 1984)

SOD (EC 1.15.1.1) activity was measured spectrophotometrically as described by Beyer and Fridovich (1987). In this assay, 1 unit of SOD is defined as the amount required to inhibit the photo reduction of nitroblue tetrazolium by 50 %. The activity of SOD was expresed as Unit/g fresh weight.

Lipid Peroxidation Levels

MDA content was determined spectrophotometrically as described by Heat and Packer (1968). 500 mg plant material was homogenized with 3 ml of 0.5% TBA in 20% TCA (W / V). The homogenized was incubated at 95 C for 30 min and the reaction was stopped in ice. The plant samples were centrifuged at 10 000 g for 15 min and absorbance of the resulting supernatant was recorded at 532 and 600 nm. The non-specific absorbance at 600 nm was subtracted from the 532 nm absorbance. The absorbance coefficient of MDA 155 mM^{-1} cm^{-1} was used in the calculation according to.

Statistical Analysis

Experimental results are mentioned as means ± SD of three parallel measurements. The statistical analysis was done by the Statistical Package for Social Science (SPSS 11.5).

3. RESULTS

10 days old seedlings of *Eruca sativa* were treated with different concentration of Mn^{+2} or Fe^{+2} added to the nutrient medium for 6 days. The effects of Fe^{+2} and Mn^{+2} on growth were expressed as dry weight of different organs of *Eruca sativa* and are shown in Figure 1. The treatment of the *Eruca sativa* plant with different concentrations of Fe^{+2} and Mn^{+2} caused significant decrease in the growth of roots and shoots. The inhibition effect of Mn^{+2} on growth was more than Fe^{+2} in both tissues of *Eruca sativa*.

The shoots and roots of *Eruca sativa* plant stressed under 5000 µM Mn^{+2} shown a decrease of %28.15 and %33.01 in dry weights at the end of 6^{th} day, respectively, when compared with control group. These values were %20.52 and %25.07 in case of stressing under 5000 µM Fe^{+2}, respectively (Figure 1).

Growth of the roots was inhibited more then shoots by the excess of Fe^{+2} and Mn^{+2} contained in the growth solution. When compared with the control group, shoot DW / root DW ratios of the both stress groups were increased with increasing the metal concentrations. The shoot DW / root DW ratios were 1.18 in the control group, 1.26 in the 5000 µM Mn^{+2} group and 1.24 in the 5000 µM Fe^{+2} group.

A chlorosis and brown spots were observed at the end of 6 days on *Eruca sativa* plant which was under Fe^{+2} and Mn^{+2} stress. In both stress groups, the total chlorophyll and carotenoid contents of *Eruca sativa* leaves were decreased at the end of 6 days when compared with the control group (Table 1- 2). The Fe^{+2}toxicity decreased the total chlorophyll content more than the Mn^{+2} toxicity. The content of carotenoid and total chlorophyll in the leaves of *Eruca sativa* plant which was treated with 5000 µM Mn^{+2} and Fe^{+2} were decreased %33.33, %26.08 for Mn^{+2} and %36.43, %29.19 for Fe^{+2} after 6 days , respectively.

.Table 1: Chlorophyll a, Chlorophyll b, total Chlorophyll and carotenoid concentrations in the leaves of *Eruca sativa* plant treated with different Mn^{+2} concentrations, after 6 day of treated. The values are mean ± SE (n = 3).

Mn^{+2} Concentrations (µM)	Chlorophyl a (mg g fw^{-1})	Chlorophyl b (mg g fw^{-1})	Chl a / b	Total Chl (mg g fw^{-1})	Carotenoids (mg g fw^{-1})
Control	3.77 ± 0.17	1.06 ± 0.04	3.55	4.83 ± 0.26	1.29 ± 0.25
10	3.58 ± 0.21	1.05 ± 0.11	3.40	4.63 ± 0.37	1.21 ± 0.21
100	3.28 ± 0.19	1.03 ± 0.07	3.18	4.31 ± 0.25	1.16 ± 0.23
500	3.16 ± 0.08	0.97 ± 0.02	3.25	4.13 ± 0.19	1.08 ± 0.18
1000	2.99 ± 0.11	0.90 ± 0.04	3.32	3.89 ± 0.29	0.92 ± 0.12
5000	2.74 ± 0.12	0.83 ± 0.01	3.30	3.57 ± 0.13	0.86 ± 0.16

Table 2: Chlorophyll a, Chlorophyll b, total Chlorophyll and carotenoid concentrations in the leaves of *Eruca sativa* plant treated with different Fe^{+2} concentrations, after 6 days treated. The values are mean ± SE (n = 3).

Fe Concentrations (µM)	Chlorophyl a (mg g fw^{-1})	Chlorophyl b (mg g fw^{-1})	Chl a/b	Total Chl (mg g fw^{-1})	Carotenoids (mg g fw^{-1})
Control	3.77 ± 0.17	1.06 ± 0.04	3.55	4.83 ± 0.26	1.29 ± 0.25
50	3.53 ± 0.09	1.05 ± 0.05	3.36	4.58 ± 0.38	1.11 ± 0.13
100	3.21 ± 0.15	1.05 ± 0.01	3.05	4.26 ± 0.22	1.05 ± 0.06
500	2.99 ± 0.07	1.03 ± 0.01	2.90	4.02 ± 0.35	0.91 ± 0.13
1000	2.83 ± 0.21	0.98 ± 0.02	2.88	3.81 ± 0.19	0.86 ± 0.17
5000	2.51 ± 0.11	0.91 ± 0.04	2.75	3.42 ± 0.21	0.82 ± 0.13

The contents of Fe^{+2}, Mn^{+2} , Cu and Zn in both shoots and roots of *Eruca sativa* plant stressed under Mn^{+2} and Fe^{+2} were determined in dry weight. Metals content were more in the roots than in the shoots. Also, the metals accumulation at the roots and shoots was increased with increasing the metals concentration. The results show that quantity of Fe^{+2} and Zn were decreased while the quantity of Cu was increased in the shoots and roots of *Eruca sativa* plant which was treated with Mn^{+2} toxicity. At the shoots of *Eruca sativa* plant treated with Fe^{+2} the quantities of Mn^{+2} and Zn were decreased while the quantity of Cu was increased. Also, in the Fe^{+2}toxicity in the roots of *Eruca sativa* plant the quantity of Mn^{+2} , Zn and Cu were decreased (Figure 2).

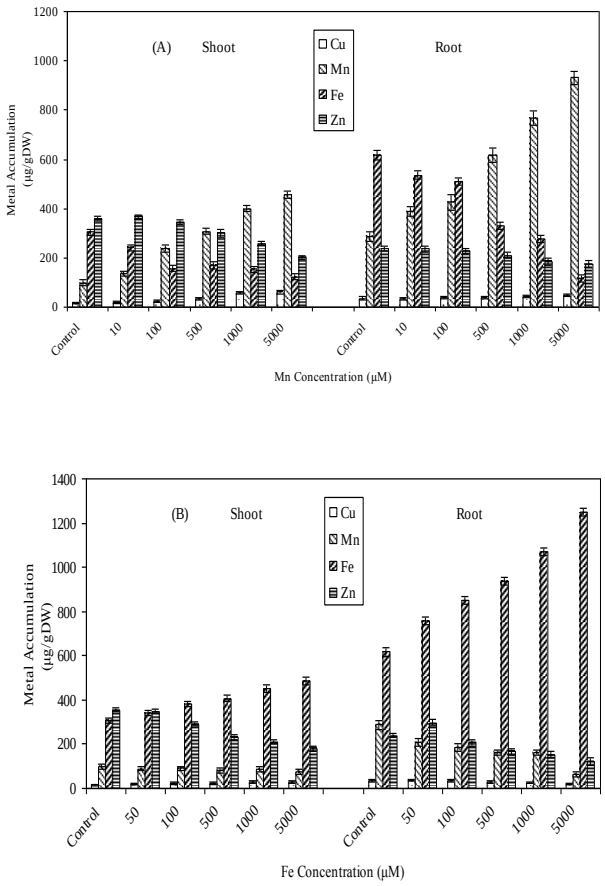

Figure 2: Metal accumulation (µg/g DW) in shoots and roots of roca plant treated with Mn^{+2} (A) and Fe^{+2} (B). The mean values of three independent experiments are shown and ± SE are indicated

The both metal stresses caused an increase in the electrolytic conductivity of the roots and shoots (Figure 1). But, the electrical conductivity of the roots was more than the shoots for the both stress groups.

When compared with the control group, the proline contents in roots and shoots of *Eruca sativa* plant were increased in the both metal stresses (Figure 1). There was not a significant difference in the accumulation of proline in the shoots and roots of plant under lower concentration of Mn^{+2} and Fe^{+2} (< 100 µM Mn^{+2} or Fe^{+2}) stress when compared with control. At higher concentration, the Fe^{+2} toxicity caused more increase in the proline content in the shoots and roots of the *Eruca sativa* when compared with the Mn^{+2} toxicity. Maximum increases in proline content of the shoots and roots of the plants stressed with Mn^{+2} or Fe^{+2} were determined 2.79 and 2.94 fold of the control for 5000 µM Mn^{+2} and 3.06 and 3.22 fold of the control for 5000 µM Fe^{+2} respectively, after 6 days treatment. There was a linear relationship between the metal accumulation and proline content in shoots and roots of the *Eruca sativa* when it was exposed to Mn^{+2} and Fe^{+2} stress (R^2=0.9102, $P < 0.01$) for Mn^{+2} toxicity in shoots, (R^2= 0.9347, $P < 0.01$) for Mn^{+2} toxicity in roots, (R^2 = 0.9643, $P < 0.01$) for Fe^{+2} toxicity in shoots, (R^2 = 0.9341, $P < 0.01$) for Fe^{+2} toxicity in roots).

It was observed that the H_2O_2 levels in the shoots and roots were increased with the intensity of Mn^{+2} or Fe^{+2} stresses. In lower concentration of Mn^{+2} or Fe^{+2} (< 100 µM Mn^{+2} or Fe^{+2}) there was not a significant difference in the H_2O_2 contents in the roots and the shoots when compared with control but the H_2O_2 levels were increased 2.59, 3.08 fold for 5000 µM Mn^{+2} and 2.22, 2.78 fold for 5000 µM Fe^{+2} in the shoots and roots of plant, respectively.

A positive correlation was observed between metal accumulation and H_2O_2 content at both tissues of *Eruca sativa* plant treated with excess Mn^{+2} or Fe^{+2} (R^2 = 0.9942, $P < 0.01$ for Mn^{+2} toxicity in shoots; R^2 = 0.9519, $P < 0.01$ for Mn^{+2} toxicity in roots; R^2 = 0.9731, $P < 0.01$ for Fe^{+2} toxicity in shoots, R^2 = 0.9763, $P < 0.01$ for Fe^{+2} toxicity in roots).

The MDA content is thought as the measure of lipid peroxidation. When compared with control group, the levels of lipid peroxidation were significantly increased at both shoots and roots of *Eruca sativa* stressed with excess of Fe^{+2} or Mn^{+2} . Peroxidation level was higher in the roots than in the shoots depending on the excess metal accumulation in the roots for both stressed groups. At the end of 6 days, the MDA levels were increased 2.36 fold in the shoots and 3.16 fold in the roots under 5000 µM Fe^{+2} stress and it was also increased 2.03 fold and 2.40 fold in the shoots

and roots of the plant respectively under 5000 μM Mn^{+2} stress (Table 3 - 4).

Increase in the SOD activity in the plant under metals stress was indicated by increasing singlet oxygen level. The SOD and GPX activities were increased at the shoots and roots of *Eruca sativa* plant which was stressed with Fe^{+2} ($P < 0.01$) and Mn^{+2} ($P < 0.01$), when compared with control groups (Table 3 – 4).

APX activity was decreased in the shoots and increased in the roots while CAT activity was increased in the shoots, and decreased in the roots of *Eruca sativa* plant which was treated with Mn^{+2} toxicity. However, both the APX and CAT activities were increased in the shoots and decreased in the roots of *Eruca sativa* plant treated with Fe^{+2} toxicity (Tablo 3 – 4).

4. DISCUSSION

Metal toxicity is one of important abiotic stress and affects some physiological and biochemical processes associated with plant growth and development (Quartin et al., 1988, Paul et al., 2003). Growth inhibition and reduction of biomass production are general responses of higher plants to heavy metal toxicity (Quartin et al., 1988).

Our results have shown that excess Mn^{+2} and Fe^{+2} inhibited plant growth. The decrease in growth may be due to the accumulation of metals in shoots and roots. Excess Mn^{+2} and Fe^{+2} greatly increased the Mn^{+2} and Fe^{+2} accumulation in the plant organs. In this study, excess Mn^{+2} significantly reduced the contents of Fe^{+2} and Zn in *Eruca sativa* shoots and roots, particularly this decrease was much greater in roots. Excess Mn^{+2} can affect unfavorably the Fe^{+2} action in tissues, catalyzing the oxidation of the physiologically active Fe^{+2+2} to the inactive Fe^{+2+3} forms (El-Jaoual and Cox, 1998).

Mn^{+2} and Fe^{+2} concentrations in roots of 5000 μM metal treated plants reflected the amount supplied to the growth media, being respectively 3.24 and 2.02 fold for Mn^{+2} and Fe^{+2} with compared control group. Mn^{+2} and Fe^{+2} concentrations in the shoots of 5000 μM metal treated plants were 4.64 and 1.59 fold higher than that of plants grown without Mn^{+2} and Fe^{+2} respectively. There was a correlation between metal accumulation and the increasing of metal concentration in growth medium in shoots ($P < 0.01$) and in roots ($P < 0.01$). Regarding to the shoots-roots distribution of Fe^{+2}, it was observed that the majority of Fe^{+2} was accumulated in the roots. The ratio between shoots and roots Fe^{+2} concentrations in 5000 μM metal treated plants was 0.388, indicating a strong metal retention in the roots. Figure 2 shows the concentrations of Cu, Zn, Fe^{+2} and Mn^{+2} in the shoots and roots of *Eruca sativa* plants supplied with different Mn^{+2} and Fe^{+2} levels. Both Fe^{+2} and Zn had similar responses to the Mn^{+2} supply a decrease in shoots and roots in all Mn^{+2} concentrations.

It has been reported that the occurrence of Mn^{+2} toxicity was closely related with Fe^{+2} concentrations in lichen tissue (Elamin and Wilcox, 1986). On the other hand, excess Fe^{+2} decreased the uptake of Mn^{+2} and Zn in both tissue of *Eruca sativa* plant. On the contrary, in Mn^{+2} and Fe^{+2} treated plant, Cu uptake in shoots and roots are increased when compared to control plants. This uptake was greater in shoots than the roots in Mn^{+2} stress.

The concentration of total chlorophyll decreased significantly in leaves in *Eruca sativa* treated with low and excess iron and manganese (Table 1 – 2). The concentration of total chlorophylls in leaves is related to the level of iron supply to the plants (Bisht et al., 2002) It has been suggested that Fe^{+2} is not directly involved in the chlorophyll biosynthesis but it is required indirectly for its biosynthesis as it controls the formation of its precursor ð-aminolevulinic acid (Marschner, 1998) or it is supposed to decrease in the role of carbon assimilation significantly affecting poor photosynthesis efficiency under Fe^{+2} stress (Sinha et al., 1997).

Table 3: Effect of excess Mn on the activities of antioxidant enzymes and lipid peroxidation of roca plant shoots and roots under drought. The values (mean ± S.E.) are calculated from three replicates.

	SOD ($U\ g\ fw^{-1}$)		CAT ($U\ g\ fw^{-1}$)		APX ($U\ g\ fw^{-1}$)		GPX ($U\ g\ fw^{-1}$)		MDA ($nmol\ g\ fw^{-1}$)	
Treatment Mn	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
Control										
1. Day	53.71 ± 3.6	83.81 ± 5.1	78.12 ± 4.3	60.38 ± 4.2	61 ± 0.04	38 ± 0.02	1.21 ± 0.02	3.51 ± 0.1	13.87 ± 1.3	15.52 ± 1.5
3. Day	55.16 ± 2.2	84.56 ± 5.3	81.46 ± 6.1	60.93 ± 3.9	73 ± 0.1	35 ± 0.02	1.12 ± 0.01	3.62 ± 0.1	15.71 ± 0.9	16.32 ± 1.9
6. Day	54.85 ± 3.2	84.43 ± 4.7	81.69 ± 6.3	61.85 ± 3.3	87 ± 0.1	25 ± 0.11	1.13 ± 0.02	3.80 ± 0.3	15.50 ± 1.2	16.05 ± 2.4
10 µM										
1. Day	54.19 ± 3.6	82.98 ± 5.6	83.70 ± 4.4	59.34 ± 2.3	71 ± 0.2	22 ± 0.09	1.16 ± 0.03	4.10 ± 0.2	14.50 ± 0.9	13.65 ± 1.3
3. Day	56.78 ± 2.9	86.19 ± 3.3	89.28 ± 6.2	56.83 ± 3.2	76 ± 0.2	01 ± 0.18	1.11 ± 0.01	5.21 ± 0.3	17.52 ± 1.2	17.14 ± 1.4
6. Day	59.62 ± 3.1	97.81 ± 4.7	92.63 ± 6.3	57.05 ± 3.4	59 ± 0.3	15 ± 0.15	1.59 ± 0.04	5.35 ± 0.2	19.61 ± 1.3	18.72 ± 1.1
100 µM										
1. Day	56.31 ± 3.6	84.53 ± 5.1	84.36 ± 5.2	56.83 ± 2.8	11 ± 0.1	52 ± 0.26	1.21 ± 0.07	4.81 ± 0.3	16.50 ± 1.6	15.50 ± 1.3
3. Day	59.32 ± 4.2	94.11 ± 6.7	93.74 ± 5.9	55.71 ± 2.5	35 ± 0.3	24 ± 0.22	1.24 ± 0.05	5.85 ± 0.5	20.12 ± 1.9	19.56 ± 1.1
6. Day	64.20 ± 3.8	101 15 ± 6.9	99.98 ± 6.3	53.83 ± 2.4	17 ± 0.4	51 ± 0.25	1.86 ± 0.07	6.12 ± 0.5	19.21 ± 1.9	20.51 ± 1.0
500 µM										
1. Day	58.15 ± 3.9	85.72 ± 6.3	91.51 ± 6.2	56.63 ± 3.1	10 ± 0.5	75 ± 0.31	1.24 ± 0.03	6.34 ± 0.4	17.22 ± 1.5	15.89 ± 1.2
3. Day	63.19 ± 3.1	97.23 ± 5.6	102.67 ± 7.2	53.04 ± 2.5	75 ± 0.3	57 ± 0.33	1.39 ± 0.04	7.11 ± 0.6	22.61 ± 2.1	23.45 ± 1.3
6. Day	69.44 ± 4.3	105 16 ± 6.8	104.23 ± 7.5	51.17 ± 2. 3	30 ± 0.3	96 ± 0.38	2.16 ± 0.08	7.64 ± 0.5	24.50 ± 1.6	26.30 ± 1.4
1000 µM										
1. Day	61.97 ± 3.9	88.19 ± 4.3	97.10 ± 5.1	54.04 ± 3.1	91 ± 0.2	55 ± 0.18	1.34 ± 0.03	7.32 ± 0.3	18.30 ± 1.7	16.90 ± 1.7
3. Day	69.11 ± 4.8	98.43 ± 4.9	106.02 ± 6.8	53.14 ± 2.7	41 ± 0.4	48 ± 0.29	1.47 ± 0.05	8.15 ± 0.7	23.75 ± 2.7	22.61 ± 2.6
6. Day	72.81 ± 4.8	107.92 ± 7.3	108.69 ± 6.1	49.12 ± 2.6	07 ± 0.3	31 ± 0.41	2.50 ± 0.08	9.31 ± 0.5	27.46 ± 2.3	31.21 ± 2.7
5000 µM										
1. Day	62.73 ± 3.5	91.78 ± 5.3	98.21 ± 6.2	57.49 ± 3.2	21 ± 0.2	86 ± 0.37	1.51 ± 0.04	7.71 ± 0.4	19.47 ± 1.1	21.50 ± 2.1
3. Day	69.92 ± 4.6	101.38 ± 6.3	110.48 ± 8.1	51.59 ± 3.5	95 ± 0.2	31 ± 0.44	1.97 ± 0.09	8.83 ± 0.6	26.51 ± 2.3	29.32 ± 2.7
6. Day	79.81 ± 5.1	115.32 ±6.8	117.18 ± 7.3	44.72 ± 3.5	10 ± 0.6	55 ± 0.39	3.29 ± 0.11	9.65 ± 0.8	31.50 ± 2.7	38.50 ± 2.6

Table 4: Effect of excess Fe on the activities of antioxidant enzymes and lipid peroxidation of roca plant shoots and roots under drought. The values (mean ± S.E.) are calculated from three replicates.

	SOD ($U\ g\ fw^{-1}$)		CAT ($U\ g\ fw^{-1}$)		APX ($U\ g\ fw^{-1}$)		GPX ($U\ g\ fw^{-1}$)		MDA ($nmol\ g\ fw^{-1}$)	
Treatment Fe (µM)	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
Control										
1. Day	53.71 ± 3.6	83.81 ± 5.1	78.12 ± 4.3	60.38 ± 4.2	4.61 ± 0.04	2.38 ± 0.02	1.21 ± 0.02	3.51 ± 0.1	13.87 ± 1.3	15.52 ± 1.5
3. Day	55.16 ± 2.2	84.56 ± 5.3	81.46 ± 6.1	60.93 ± 3.9	4.73 ± 0.1	2.35 ± 0.02	1.12 ± 0.01	3.62 ± 0.1	15.71 ± 0.9	16.32 ± 1.9
6. Day	54.85 ± 3.2	84.43 ± 4.7	81.69 ± 6.3	61.85 ± 3.3	4.87 ± 0.1	2.25 ± 0.11	1.13 ± 0.02	3.80 ± 0.3	15.50 ± 1.2	16.05 ± 2.4
10 µM										
1. Day	53.52 ± 2.1	83.90 ± 5.3	91.51 ± 7.1	58.83 ± 3.12	4.19 ± 0.21	2.19 ± 0.13	1.28 ± 0.03	3.72 ± 0.2	15.50 ± 2.6	13.18 ± 2.2
3. Day	55.32 ± 3.2	83.23 ± 5.2	139.04 ± 9.2	58.93 ± 2.98	4.88 ± 0.17	2.50 ± 0.21	1.32 ± 0.02	3.91 ± 0.2	20.53 ± 2.5	15.27 ± 2.6
6. Day	57.71 ± 2.6	94.12 ± 6.1	142.84 ± 13.8	60.11 ± 3.21	4.33 ± 0.06	2.68 ± 0.42	1.44 ± 0.01	4.18 ± 0.3	22.17 ± 2.1	18.50 ± 2.9
100 µM										
1. Day	55.61 ± 3.1	84.78 ± 4.3	95.97 ± 5.89	58.28 ± 2.48	4.92 ± 0.23	1.87 ± 0.18	1.12 ± 0.02	3.82 ± 0.3	17.46 ± 3.2	15.26 ± 2.5
3. Day	57.94 ± 3.4	91.26 ± 4.7	133.02 ± 12.6	57.93 ± 3.01	5.75 ± 0.19	1.34 ± 0.11	1.45 ± 0.01	4.11 ± 0.4	21.28 ± 3.1	19.49 ± 3.3
6. Day	61.56 ± 3.7	99.36 ± 5.4	147.31 ± 11.3	54.93 ± 1.98	4.34 ± 0.31	1.71 ± 0.07	1.72 ± 0.01	4.56 ± 0.2	27.50 ± 2.1	23.50 ± 3.5
500 µM										
1. Day	57.22 ± 2.7	85.17 ± 5.6	117.62 ± 9.6	56.72 ± 2.16	4.93 ± 0.07	1.64 ± 0.13	1.41 ± 0.02	3.93 ± 0.3	18.06 ± 2.3	17.56 ± 3.1
3. Day	62.18 ± 2.8	93.81 ± 7.1	151.77 ± 11.8	55.38 ± 1.87	5.40 ± 0.21	2.45 ± 0.08	1.72 ± 0.04	4.17 ± 0.2	25.50 ± 2.8	27.47 ± 4.1
6. Day	65.22 ± 3.3	102.92 ± 7.0	158.47 ± 9.3	55.11 ± 2.40	4.82 ± 0.33	1.58 ± 0.12	2.12 ± 0.02	4.45 ± 0.3	30.07 ± 3.1	35.06 ± 3.3
1000 µM										
1. Day	58.39 ± 3.7	86.69 ± 4.6	134.58 ± 12.9	55.72 ± 2.02	5.43 ± 0.39	1.41 ± 1.35	1.94 ± 0.03	4.13 ± 0.2	18.13 ± 2.2	19.13 ± 3.2
3. Day	64.67 ± 4.3	94.19 ± 6.2	182.57 ± 15.8	56.83 ± 3.10	6.29 ± 0.68	2.72 ± 0.27	2.04 ± 0.05	4.88 ± 0.3	25.73 ± 2.5	27.53 ± 3.5
6. Day	68.89 ± 4.8	104.33 ± 7.9	190.17 ± 14.7	52.83 ± 3.61	6.27 ± 0.71	1.23 ± 0.19	2.64 ± 0.04	5.11 ± 0.5	34.45 ± 2.3	41.75 ± 4.6
5000 µM										
1. Day	60.21 ± 4.9	88.12 ± 6.3	151.99 ± 10.4	53.72 ± 2.03	4.80 ± 0.33	1.58 ± 0.08	2.96 ± 0.07	4.36 ± 0.3	26.53 ± 3.1	26.32 ± 2.6
3. Day	68.56 ± 3.7	97.27 ± 5.2	195.07 ± 14.4	51.83 ± 2.11	6.01 ± 0.48	2.11 ± 0.12	2.43 ± 0.05	5.10 ± 0.4	31.23 ± 2.6	34.21 ± 2.7
6. Day	75.44 ± 5.3	106.2 ± 7.0	207.57 ± 16.9	47.83 ± 2.31	8.77 ± 0.51	1.19 ± 0.09	3.15 ± 0.11	6.47 ± 0.5	36.60 ± 3.2	50.73 ± 5.1

Mn^{+2} is toxic when in excess and consequently it represents an important factor in environmental contamination with various phototoxic effects. Mn^{+2} toxicity is often more common than Mn^{+2} deficiency in acid soils for a number of plant species (Foy et al., 1988, Hue et al., 2001). Mn^{+2} induced Fe^{+2} deficiency is known to be a possible cause for reduced chlorophyll concentrations in vascular plants, as Fe^{+2} is essential for chlorophyll biosynthesis (Horst, 1988, El-Jaoual and Cox, 1998). A typical symptom of Mn^{+2} toxicity in vascular plants is intervenial chlorosis resembling Fe^{+2} deficiency (Ohki, 1984, Foy et al., 1988). This is because Mn^{+2} is known to play an important role in the biosynthesis of chlorophyll and several enzymes along the isoprenoid biosynthetic pathway (Wilkinson and Ohki, 1988). Thus excess Mn^{+2} inhibits chlorophyll synthesis, induces a decline in photosynthetic rate (Macfie and Taylo, 1992). Excess Mn^{+2} reduces the concentrations of the chlorophylls a and b in the epiphytic lichen *Hypogyia physodes* (Hauck et al., 2003)

It has been reported that some metal compounds have high potential to promote oxygen free radical production and thus lead to peroxidation of membrane lipids. Some metals like Cu, Fe^{+2}, Mn^{+2} and Cd are known to induce peroxidation of lipids in plants (De Vos et al., 1989; Çakmak and Host, 1991; Sinha et al., 1997; Shah et al., 2001). Similar to these reports excessive uptake of iron and manganese in *Eruca sativa* plants resulted in an accumulation of lipid peroxidation product (LPO levels). The treatment with Fe^{+2} and Mn^{+2} induced a significant increase in H_2O_2 content of *Eruca sativa* both in roots and shoots in comparison with the control. Stress conditions enhance H_2O_2 production in different compartments of plant cells by enzymatic and non enzymatic process (Foyer et al., 1994).

In the present study, the significant increase in electrolytic leakage with increase iron and manganese concentrations is in agreement with the findings of Sinha et al. (1997). Fe^{+2} treated plants exhibited an increase in K^+ leakage (electrolytic leakage) as well as an increase in lipid peroxidation product (Sinha et al., 1997). Lipid peroxidation profoundly alters the structure of membranes and consequently modifies their enzymatic and transport activities. A well-known harmful effect of metals Hg, Cu and Cd is the alteration of plasma membrane permeability of cells leading to the leakage of potassium ions (De Vos et al., 1989, Shah et al., 2001).

Proline is known to accumulation under heavy metal exposure and considered to involve in stress resistance (De Vos et al., 1989, Sinha et al., 1997). Our studies show that Fe^{+2} and Mn^{+2} were effective in inducing proline accumulation in roots and shoots of *Eruca sativa* plant. Proline accumulation was reported in rice and *Ceratophyllum demersum* subjected to metal stress (Devi and Prasad, 1998; Molassiotis et al., 2005).

The individual enzymes measured in the present study showed variable responses to Fe^{+2} and Mn^{+2}, such that both induction and inhibition of enzyme activities by metals occurred. Differential responses of shoots and roots enzymes to stress have been observed for all other enzymes measured. In plant organs, stimulation of some detoxifying enzymes during metal treatment might result from an induction of these enzymes through the production of O_2^- and H_2O_2 (Prasad et al., 1994). Different studies demonstrated that metal tolerance in various species was partly the result of an enhanced antioxidant defense system (Devi and Prasad, 1998).

However, differential responses to Mn^{+2} and Fe^{+2} treatments were observed only for APX in roots and shoots, while no significant differences in the effected of Mn^{+2} and Fe^{+2} treatments on SOD, GPX and CAT activities in shoots and roots were found. These results confirm the notion that changes in antioxidant activities represent an unspecific response to oxidative stress; therefore, the activity of antioxidant enzymes many serve only as a general indicator of plant stress (Bowler et al., 1992).

In plants, the detoxification of H_2O_2 has been known to be an important function of the

peroxidase that use ascorbate as the hydrogen donor (Hegedüs et al., 2001). APX is believed to scavenge excess H_2O_2 formed in plant cells under normal and stress conditions (Nakano and Asada, 1981) and many authors have reported enhanced expression of APX in response to environmental and biotic stresses (Devi and Prasad, 1998). Though transition metals like Fe^{+2} and Cu are know to cause oxidative injury in plant tissues, non-redox reactive metals like Al also result in oxidative stress as indicated by H_2O_2 accumulation and oxidative burst (Schutzendubal and Polle, 2002, Boscolo et al., 2003). The APX activity was found to increase in the roots of Mn^{+2} treatment *Eruca sativa* and in the shoot of Fe^{+2} treatment *Eruca sativa*. The induction of APX activity in plants was also reported in *Ceretophyllum demersium* under copper stress (Devi and Prasad, 1998) and Cu treated *phaseolus vulgaris* (Gupta et al., 1999).

Furthermore H_2O_2 has been shown to induce cytosolic APX (Morito et al., 1999). Therefore, the H_2O_2 accumulation under high metal concentrations may be a signal for adaptive response to the stress (Foyer et al., 1994, Van Breusegem, 2001).

While the CAT activity was found to increased in shoots both of Mn^{+2} and Fe^{+2} treatment *Eruca sativa* plant, its decreased in roots. An increase in the activity of catalase has been reported in certain plant species exposed to toxic concentrations of heavy metals Cu, Fe^{+2}, Zn (Devi and Prasad, 1998; Gupta et al., 1999; Rout et al., 1999). However under a variety of environmental stresses like salinity chilling drought, an overall decline in catalase activity was observed (Morito et al., 1999). Such protective enzymes are activated in plants which stimulate production of oxygen free radicals. The superoxide anion radical is disproportionate spontaneously by SOD to produce H_2O_2.

Superoxide dismutase is an important defense system of plants against free radicals. Our results show increase in the activity of SOD in *Eruca sativa*. With increase in the levels of Fe^{+2} and Mn^{+2} in growth medium, SOD activity has been reported to be stimulated under variety of stressful conditions. The increase in SOD activity in response to stresses is possibly attributed to the de-novo synthesis of the enzymatic protein (Beyer and Fridovich 1987). Similar results have been registered in plants to Mn^{+2} , Fe^{+2} and other metal toxicity (Devi and Prasad, 1998, Rout et al., 1999, Van Breusegem, 2001). Therefore, increase in SOD activity may be considered a circumstantial evidence for enhanced production of oxygen free radicals (Beyer and Fridovich 1987).

Among H_2O_2 destroying enzymes, GPX activity was found to increase in roots and shoots of the both Mn^{+2} and Fe^{+2} treated *Eruca sativa*. Shah et al. (2001) also reported similar finding showing increase in GPX activity in the metal (Cd) treated plants. The increase in the activity of GPX levels could be the consequence of their micro-environment or the tissue specific gene expression in the treated plants (Hegedüs et al., 2001). An increase in GPX activity could be considered not only as a reaction to metal-caused oxidative damage (Devi and Prasad, 1998; Fe^{+2}cht-Christoffer et al., 2003), but also as a common response to various stress factors (Janda et al., 2003). Under excess Mn^{+2} or Fe^{+2} stress conditions significant elevation in GPX activity showed its role in constant detoxification of H_2O_2 in *Eruca sativa* shoots and roots.

In conclusion, because ROS generation was closely related with Fe^{+2} and Mn^{+2} stress, in response to this, increases in the proline content, LPO levels, H_2O_2 accumulation, electrolytic leakage and in the activities of some antioxidant enzyme activities (SOD, GPX) were observed in plant organs of *Eruca sativa* when compared to control plants. The presented showed that higher Mn^{+2} or Fe^{+2} concentrations might promose the oxidative damage to membrane.

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