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Iron Deficiency Stimulated Some Enzymes Activity, Lipid Peroxidation and Free Radicals Production in *Borage officinalis* Induced *in vitro*

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ABSTRACT

Iron deficiency is one of the most important factors affecting growth and yield. Utilizing tissue culture technique to study the response of plant to Fe-stress was suggested as a rapid and simple method. Fe-deficiency was explored by cultivating shoots of Borage plantlets on MS media with 27.8, 13.0, 0.0 mg/L $FeSO_4$. Results showed that, severe Fe-deficiency induced yellowing and typical iron deficiency symptoms. Chlorophyll was also inhibited. The response of antioxidant enzymes, such as catalase (CAT) and ascorbate peroxidase (APX) to iron stress was also examined. CAT and APX activities declined by 49.3% and 56.6% in young Fe-deficient leaves respectively. Whereas the content of glutathione increased by 28.9% compared to control treatment. Enhancement in lipid peroxidation was also more evident under severe Fe-deficiency. Iron stress also induced several changes in CAT and peroxidase (POD) isozyme profiles and enhanced their activities, especially those of POD. Using electron spin resonance (ESR) showed that, severe Fe-deficiency markedly increased level of free radicals formation.

Key Words*: Borage officinalis*; Ascorbate Peroxidase; Catalase; Electron Spin Resonance; Iron Deficiency; Isozymes profile

INTRODUCTION

Iron is considered as an essential nutrient for plant growth and plays a central role on overall physiology of the plants. When plants subjected to iron deficiency stress a range of deleterious effects including inhibition of photosynthesis processes, pigment synthesis and other metabolic disturbances such as change in the levels of several metalloenzymes activity were established (Terry, 1980; Manthey & Crowely, 1997).

The relationship between decreased iron availability in the nutrient media and the possible onset of oxidative stress is becoming more evident, because of the dual role played by iron in cell metabolism as either an antioxidant or a pro-oxidant factor. In fact, iron is a constituent or a cofactor of many antioxidant enzymes but, on the other hand, it can act as pro-oxidant because it catalyses free radicals generation through the Fenton's reaction (Minotti & Aust, 1987) .However, it has been shown that many enzymes require iron in order to function correctly ;in particular iron is present in the active sites of catalase and super- oxide dismutase involved in the scavenging of reactive oxygen species (ROS) as described previously by Elstner and Osswald, (1994).

The generation of ROS such as superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\bullet}OH$), can damage many cellular components including proteins, membrane lipids and nucleic acids, so plant cells respond to the formation of ROS by increasing the production of metalloenzymes such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), peroxidase (POD, EC 1.11.1.7) and especially ascorbate peroxidase (APX, EC 1.11.1.6) that protecting cells against oxidative injury caused by many biotic and abiotic stresses (Halliwell & Gutteridge, 1989) .In plants, several isomeric forms of metalloenzymes catalyses the dismutation of the superoxide radical $O_2^{\bullet -}$ to H_2O_2 were detected and the change in isoenzyme profiles might provide an added protective role in response to environmental stress (Pinhero *et al*., 1997). Also, antioxidative enzymes occur in cells in many isoforms, often exhibiting different properties and thereby limit the formation of (ROS) generation (Noctor & Foyer, 1998). The electron spin resonance (ESR) studies, involving spin trapping techniques to detect short-lived free radicals, are able to detect the formation of free radicals in plants during micronutrient stress such as Zn deficiency (Cakmak & Marschner, 1988). The ESR method can also be utilized for estimating the type and content of more stable free radicals that accumulate *in vivo* as markers of oxidative stress due to O_2 depletion and enrichment (Goodman *et al*., 1986).

The objective of this work is to demonstrate if the activities of antioxidant enzymes, isozyme profiles, glutathione content and lipid peroxidation, together with free radical formation in Borage plantlet can be used to evaluate plant response to iron stress using *in vitro* culture

technique as a potentially more rapid methodology. The procedure adopted for this study was that of decreasing Fe availability in the controlled environment of culture media.

MATERIALS AND METHODS

Plant culture. Borage (*Borage officinalis* L.) seeds were surface sterilized by 70% ethanol followed by 0.5% Na-hypochlorite (30% Clorox) finally the seeds were rinsed with sterile distilled water. Seeds were immediately cultured in MS-media according to Murashige and Skoog (1962) supplemented with vitamins, 100 mg/L inisitol, 30 g/L sucrose and 2.0 g/L gerlite. The pH of the medium was adjusted to 5.7, and then the media was autoclaved. All cultures were kept for 4 weeks in a controlled environment conditions according to Janick *et al.* (1987). The trial was set up after the fourth subculture. The shoot tips of the regenerated plantlets were transferred to new MS media and subjected to three iron treatments as follows: (i) control plants, Fe was supplied as $FeSO_4$ at a concentration of 27.8 mg/L or (ii) 13 mg/L [mild Fe deficiency] (iii) no iron was added to the media [severe Fe deficiency]. After four weeks the regenerated plantlets of each treatment were used for the following analysis.

Iron determination. The leaves of intact plantlets were washed by deionized water and the tissue was oven dried at 90°C. Oven dried tissue ashed at 500°C then the ash was dissolved in 1% w/v HCl. Total iron was determined using atomic absorption spectroscopy (Perken Elmer model) according to the method of Jones (1984).

Chlorophyll determination. Chlorophyll (Chlorophyll *a* and *b*) was extracted from fresh leaf tissue with 80% acetone, then the absorbency was determined at 645 and 663 nm with LKB spectrophotometer and chlorophyll concentration were (mg/g f.wt.) calculated according to the equations of Lichtenthaler (1987).

Protein extraction. Soluble protein extracts were prepared at 4°C and homogenized in ice-cold 250 mM sucrose buffer (pH 7.2) containing 1% PVP-40 and 1 mM EDTA in a chilled mortar and pestle. The homogenate was filtered and centrifuged at 12,500 rpm for 20 min at 4°C. The obtained supernatant was used for measurement of enzyme activity, peroxidase and catalase isozyme. Protein content was determined spectrophotometrically at 595 nm, using the method of Bradford (1976). Bovine serum albumin (BSA) was used as a protein standard.

Ascorbate peroxidase determination. APX activity was determined by the method given by Nakano and Asada, (1981). The APX activity was measured as a decrease in absorbance at 290 nm due to oxidation of added ascorbate during 1 min period. ($\sum 290 = 2.8\ mM^{-1}\ cm^{-1}$).

Catalase determination. CAT activity was assayed at 240 nm by the method given by Chance and Maehly (1955) in a total volume of 1.0 ml of 25 mM K-phosphate buffer pH 6.8, 10 mM H_2O_2 and a diluted enzyme extract.

Glutathione determination. The level of glutathione was determined with Ellman's reagent as described by Tukendorf and Rauser, (1990). Three- hundred microliters of supernatant was mixed with 630 µL of 0.5 M K_2HPO_4 and 25 µL of 10 m M 5,5-di thiobis (2- nitrobenzoic acid) final pH 7. The absorbance at 412 nm was read after 2 min ($\sum 412 = 13.6\ mM^{-1}\ cm^{-1}$).

Isozymes electrophoresis. Non denaturing polyacrylamide gel electrophoresis (PAGE) took place according to the method of Davis, (1964),each well contain equal amount of proteins(30µg) Activity stain for enzymes was carried out as follows: For peroxidase (POD) isozyme, gel was stained with O-dianisidine as described by Amako *et al.* (1999).

Catalase (CAT) isozyme activity was localized by incubating the gel in a reaction mixture containing 1% potassium ferricyanide and 1% ferric chloride, based on the method of Woodbury *et al.* (1971).

Lipid peroxidation (TBA test). The level of lipid peroxidation in the tissues was determined as 2-thiobarbituric acid (TBA) reactive metabolites chiefly malondialdehyde (MDA) as described by Buege and Aust (1978). The absorbance of the pink color was measured at 532 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The concentration of MDA was calculated based on $A_{532} - A_{600}$ ($\Sigma = 155\ mM^{-1}\ cm^{-1}$).

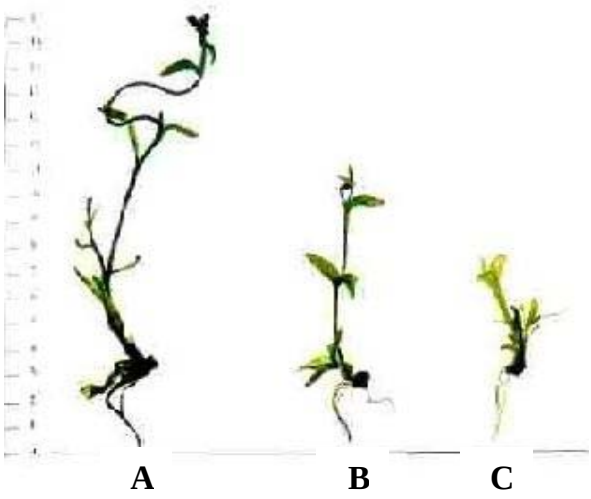
The spectra of electron spin resonance (ESR). Representative sample composed of 10 roots from each treatment were excised on ice and stored at –20°C. Frozen samples were lyophilized, and were then ground in a mortar. The powdered material (Ca. 500 mg) was packed into ESR flat quartz cuvette. The ESR spectra were recorded at room temperature using ESR spectrometer ELEXSYS E500 (Bruker-Germany) model. The experimental condition was as follows: Field Mod. Amplitude 0.001, Field Mod. Frequency (Hz) 100000, Microwave Frequency (Hz) 9.80078e+09, Microwave power (W) 0.000640794, Receiver Gain 50, Receiver Time constant (S) 0.00512.

The standardization of "g" value was carried out using 1,1 Diphenyl –2- picryl-hydrazyl (DPPH) as a standard. The areas under ESR curve for each sample were calculated. The area under the curve of the control sample was represented as 100% and was used to plot the relative radicals versus iron concentration.

RESULTS AND DISCUSSION

Plant growth. Borage plantlets grown in the absence of Fe in the growth media developed typical Fe- deficiency symptoms such as young leaves showed yellowing and dwarfing due to inhibition of cell division, thus leaf growth is impaired (Fig. 1). Also whole roots and root tips were yellow, due to the accumulation of riboflavin (Susin *et al.*, 1993). In contrast, plantlets grown on the control media showed no symptoms of chlorosis throughout the experiment period. With the onset of visual Fe-deficiency

Fig 1. Plantlets of *Borage officinali* obtained in *in vitro* after 4 weeks from starting the Fe treatments.
(A) Control; (B) Mild Fe –deficiency; (C) Severe Fe - deficiency



symptoms, plantlets showed increasing chlorosis symptoms with Fe level in culture media, there is an inhibition in shoot growth, which reached 64.7% and 41.2% in case of mild and severe Fe- deficiency respectively compared to control treatment as shown in (Fig. 1). This inhibition might be due to the disturbances of cell division (Abbott, 1967), thus leaf growth is impaired. Borage plantlets subjected to Fe-deficiency showed yellowing due to inhibition in chlorophyll synthesis.

Iron and chlorophylls concentration. The level of iron concentration in leaf tissue was significantly affected by iron stress. The absence of iron in the growth media [mild and severe Fe deficiency] resulted in a high decrease in iron concentration in the leaves of Borage, 51.6% and 79.9% for plantlets in the complete nutrient media (Table I). In addition chlorophylls concentration were drastically reduced gradually as Fe level decreased in growth media Under severe Fe-deficiency the leaves contained less chlorophyll *a* and *b* compared with control treatments (Table I). The lower chlorophyll concentration can be explained, at least in part, by the role of Fe in the formation of δ - aminolevulinic acid and protochlorophyllide, precursors of the chlorophyll molecule (Marschner, 1986). Iron deficiency caused 36.2% drop in the content of total chlorophylls. This result is in agreement with Monterio and Winterbourm (1988) who explained the decrease of chlorophylls content by stimulation of chlorophyll oxidation due to excessive free radical formation under iron stress. In addition Lombardi *et al*. (2002) reported that, the plantlets of peach rootstock grown in the absence of iron showed a severe chlorosis due to a reduction in chlorophyll and carotenoids concentration. Moreover, when the plantlets adequately supplied with iron in the growth media, the iron concentration in leaves tissue reached 104.1 mg / kg d.w while the corresponding value under severe Fe-deficiency treatment reached 20.9 mg/kg d.w. Also Manthey and Crowley (1992) obtained similar results in Avocado plant.

Antioxidant enzymes, glutathione and lipid peroxidation. The enzymatic activities, glutathione level and lipid peroxidation found under control treatment were set to 100% and used to calculate relative change found in each other treatment (Table II). Generally, the enzymes activity were inhibited by iron deficiency, activity of CAT and APX decreased by 49.3% and 56.6% respectively under severe Fe deficiency treatments compared to control treatment. This trend may attribute with the result of less Fe concentration in Fe-deficient leaves.

Glutathione that maintains the cellular redox status showed an increase in its level under iron deficiency (Table II). As compared to the control, the decrease was more pronounced under severe Fe deficiency (28.9%) and mild (63%) respectively. The increased accumulation of lipid peroxidation is indicative of enhancement of production of toxic oxygen species. As a whole the level of MDA (one of the major TBA reaction metabolites) increased in iron deficient Borage plantlets. The increase in MDA content was more significant at severe than mild Fe deficiency treatments. In control leaves low MDA content were found, amounting to 6.5 ± 1.09 nmol MDA/mg protein/h (n=3) and 18.6 ± 0.39 nmol MDA/mg protein/h (n=3) in severe Fe-deficiency leaves, thus iron deficiency enhanced lipid

Table I. Iron and chlorophylls concentration in leaves of Borage plantlets grown with different level of iron supply in *in vitro* culture

Treatments	Fe-Conc. (mg/kg d.w)	Chlorophylls (mg/g f.w)		
		Chl. a	Chl. b	Total
Control	104.1 ± 0.17	0.63 ± 0.38	0.42 ± 0.23	1.05 ±0.43
Mild Fe-deficiency	50.4 ± 0.24	0.49 ± 0.16	0.32 ± 0.1	0.81 ± 0.28
Severe Fe-deficiency	20.9 ± 0.15	0.38 ± 0.12	0.29 ± 0.1	0.67 ± 0.21

Values given are average of at least three-replication ± S.D.

Fig. 2. Native gel stained for activity of POD in leaves of plantlets of *Borage officinali* grown in *in vitro* culture. Lanes (A, B, C) for control, mild and severe iron treatments respectively

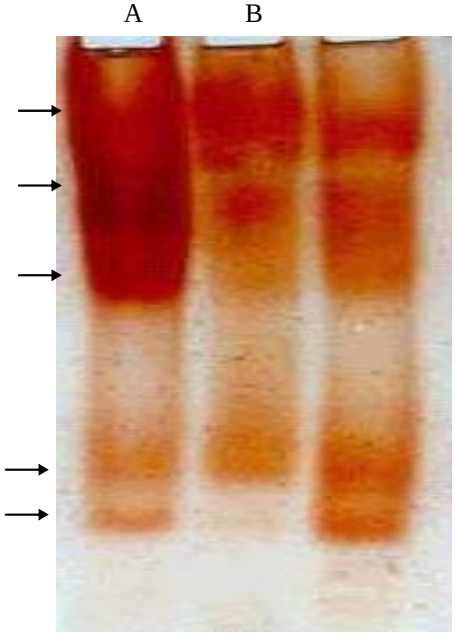
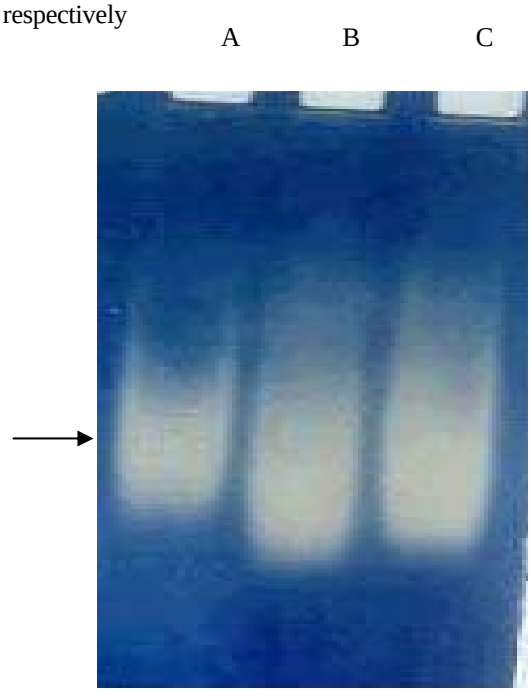


Fig. 3. Native gel stained for activity of CAT in leaves of plantlets of Borage officinal grown in *in vitro* culture. Lanes (A, B, C) for control, mild and severe iron treatments respectively



peroxidation .There is a very strong correlation between the presence of iron and the activity of the metalloenzymes; the maintenance of the normal levels of iron is essential to guarantee the activity of such antioxidant enzymes. In leaves of Borage plantlets, the specific activity of CAT and APX were reduced under Fe deficiency. These enzymes are a hemoprotein and the decrease in its activity is reflecting a depletion of the available Fe pool in the root cell. It has been reported that the activity of APX decreased under stress conditions, including Fe-deficiency in sugar beat (Zaharieva & Abadia, 2003). Determination of leaf peroxidase activity in field grown avocado trees showed that the dependence of peroxidase activity levels on Fe concentrations was statistically significant (Manthey & Crowley, 1997). The consistent decline in catalase activity might be conductive to an increase in the concentration of H_2O_2 in leaves. (Halliwell & Gutteridge, 1989). Catalase and peroxidase activities were correlated with the Fe content in leaves. These findings indicate that under severe Fe deficiency the activity of these haemoproteins is determined by Fe availability and is an excellent marker of the Fe-responses. GSH are also very important in plant cell. Present results indicate that Fe deficiency treatment increase the concentration of GSH level, this metabolites and enzymes activity is known to participate in cell antioxidant defense. In addition Astolfi *et al*. (2002) reported that iron deprivation lead to an increase in the level of non-protein sulfhydryl (SH) compounds in maize plants.

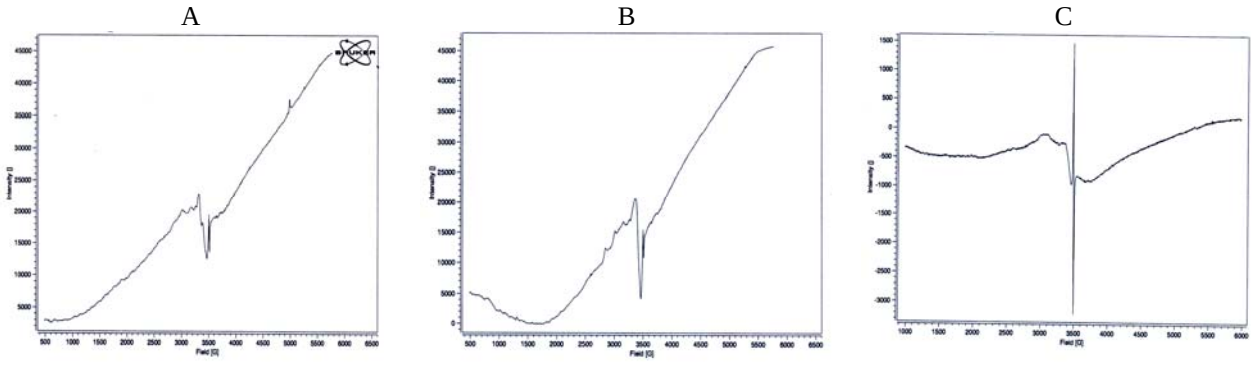
Peroxidase isoenzyme. When protein extracts from leaves were separated by native electrophoresis and monitored for POD activity (Fig. 2) the electrophoretic patterns showed differences in the number and the intensity of the bands among iron treatments. Under the control condition, electrophoretic patterns were

Table II. Activities of CAT, APX, GSH level and MDA content in leaves of Borage plantlets grown with different level of iron supply in *in vitro* culture

Treatments	CAT Sp.activity	%*	APX Sp.activity	%*	GSH Level	%*	TBA MDA	%*
Control	127.9± 3.2	100.0	80.5± 1.2	100.0	7.5± 0.29	100.0	6.5± 1.09	100.0
Mild Fe-deficiency	102.1± 2.9	80.1	60.8± 1.5	75.5	8.7±0.13	63.0	8.5 ± 0.25	130.8
Severe Fe- deficiency	63.1± 2.5	49.3	45.6± 2.3	56.6	13.8 ±0.09	28.9	18.6 ± 0.39	286.2
Severe Fe-deficiency/ control	0.49		0.57		1.89		2.9	

Specific activity of CAT: µmol H_2O_2/ mg pro/min; Specific activity of APX = µmol ascorbate oxidised/ mg pro./min; GSH = µmol /g f.w; TBA – MDA = nmol; malondialdihyde/ mg pro./h; * % Relative to control; Values given are average of at least three replication ± S.D.

Fig. 4. Electron spin resonance (ESR) spectra of free radicals formation, the 3 spectra were as follows: A, control Fe; B, mild Fe-deficiency; C, severe Fe-deficiency



characterized by the appearance of three main distinct bands with relative mobility of 0.21, 0.28 and 0.42. In addition the same treatment led to appearance of additional two bands with relative mobility 0.78 and 0.85. Under mild Fe stress, low iron concentration caused disappearance of one band with RF value 0.85. Therefore, the absence of this band might consider as a response to iron stress. It is also interesting to note that under severe Fe deficiency the electrophoretic bands showed difference in its mobility and intensity of the bands, compared to control treatment**.**

Catalase isoenzme. Evidence of the strong induction of catalase during Fe-deficiency in Borage leaves extract is shown in Fig. 3. The soluble proteins fractions under control and iron stress treatments showed strong catalase activity which appeared in one band which migrated towards the middle part of the gel with high intensity.

Synthesis of new isozymes of antioxidant enzymes may be mere enhancement of the activities of existing antioxidant enzymes (Edwards *et al.*, 1994; Rao *et al.*, 1996). Isozyme profiles of POD were varied in their response to iron stress, whereas CAT profiles showed only one band with different mobility and intensity. This effect may play an additional role in plant stress response mechanisms.

Leidi *et al*. (1989) reported that peroxidase isozyme patterns of soybean plants grown in nutrient solution are affected by iron composition of the solution. In addition De Varennes and Carvlho (1993) found that toxic levels of manganese, boron, zinc, copper, cobalt, molybdenum or iron can lead to the appearance of new zymoraphic forms or to the inhibition of isozymes present in non-stressed white lupin plants.

The spectra of ESR. The direct detection of stable free radicals by ESR spectroscopy in roots isolated from control and Fe-stressed plantlets revealed that at control Fe treatment, the level of stable free radicals production was similar to those of mild Fe deficiency treatment. These levels, however, greatly increased at a severe Fe deficiency (Fig. 4). The value of beak area (which is related to the concentration of free radicals) was: 427.12; 458.6; 1080.0, respectively.

An enhanced level of lipid peroxidation in leaves of Borage plantlets grown under Fe- deficiency indicate that iron deficiency caused oxidative damage to plantlets. Lipid peroxidation in leaves may be a consequence of generation of ROS as evident from the increased ESR spectra (Fig. 4) in Fe stress treatment.

Both redox active (Cu and Fe) and non-redox active metal ions (Zn) are reported to increase lipid peroxidation via ROS generation in plants (Shaw, 1995; Gallego *et al.*, 1996). The ESR method made it possible to quantitative changes in free radicals concentration in root tissue under control and Fe treatment .The arbitrary intensity, which is related to the concentration of free radicals, increased with increasing of Fe-deficiency. The change in amount of an $O2^{\cdot -}$ measured directly by ESR spectroscopy was related to Zn deficiency (Cakmak, 2000).

CONCLUSION

The results presented here suggest that *in vitro* systems could yield useful information to elucidate plant response to Fe-deficiency. Tests on *in vitro* grown whole plants have the advantages of small scale with clear visibility for monitoring both shoot and root response in the presence of imposed stress, but further tests will be necessary.

REFERENCES

Abbott, A.J., 1967. Physiological effects of micronutrient deficiencies in isolated roots of *Lycopersicon esculentum*. *New Phytol*., 66: 419–37

Amako, A., K. Chem and K. Asada., 1999. Separate assays specific for ascorbate peroxidase and guiacol peroxidase and for the chloroplastic and cytosolic isozyme of ascorbate peroxidase in plants. *Plant Cell Physiol*., 32: 497–504

Astolfi, S., S. Zuchi and C. Passera., 2002. Sulfate metabolism in Fe–deficient maize plants. *In: XI Int. Sym. on Iron Nutr. and Interactions in Plant.* p. 97. University of Udiue– Italy, 23–28 June

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochem.*, 72: 248–54

Buege, J.A. and S.D. Aust., 1978. Microsomal lipid peroxidation. *Methods in Enzymol.*, 52: 302–12

Cakmak, I., 2000. Possible roles of zinc in protecting plant cells from damage by active oxygen species. *New Phytol.*, 146: 185–205

Cakmak, I. and H. Marschner, 1988. Zinc–dependent changes in ESR signals, NADPH oxidase and plasma membrane permeability in cotton roots. *Physiol. Plant.*, 37: 182–6

Chance, B. and A. Maehly, 1955. Assay of catalases and peroxidases. *Methods in Enzymol.*, 2: 764–75

De Varennes, A. and L. Carvalho, 1993.Trace element and isoenzyme activities in white lupin. *In:* Fragoso, M. and M.L. Van Beusichem, (ed.). *Optimization of Plant Nutrition.* pp: 471–6. Kluwer Acad. Pub., The Netherlands

Davis, B.J., 1964. Disc electrophoresis–II method and application to human serum proteins. *Ann. New York Acad. Sci*., 121: 404–27

Edwards, E.A., C. Enard, G.P. Creissen and P.M. Mullineaux, 1994. Synthesis and properties of glutathione reductase in stressed peas. *Planta*, 192: 137–43

Elstner, F. and W. Osswald., 1994. Mechanism of oxygen activation during plant stress., *Proc. R. Sci. Edinb*., 102B: 131–54

Gallego, S.M., M.P. Benavides, and M.L. Tomaro, 1996. Effect of heavy metal ion excess on sunflower leaves: evidence for involvement of oxidative stress. *Pl. Sci.*, 121: 151–9

Goodman, B.A., D.B. McPhail and D.J. Linehan, 1986. Oxygen induced free radical in wheat roots. *Free Radic., Res., Commun*., 2: 173–8

Halliwell, B. and J.M. Gutteridge, 1989. Protection against oxidants in biological system: the superoxide theory of oxygen toxicity. *In:* Halliwell, B. and J.M. Gutteridge (eds.). *Free Radicals in Biology and Medicine.* pp: 86–123. Clarendon Press, Oxford

Janick, J., J.E. Simon and A. Whipkey, 1987. *In vitro* propagation of Borage. *Hortsci.*, 22: 493–5

Jones, J.B., 1984. Plants. *In:* Williams, S. (ed.). *Official Methods of Analysis of the Association of Official Analytical Chemists.* pp: 38–64. Arlington, Virginia, USA

Leidi E.O. and M. Gomez, 1989 Peroxidase isozyme patterns developed by soybean gentypes in response to manganese and iron stress. *Biochem. Physiol. Pflanz*, 185: 391–6

Lichtenthaler, H.K., 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Methods in Enzymol*., 148: 350–82

Lombardi, L., L. Sebastiani and C. Vitagliono, 2002. Physiological, biochemical and molecular effects of *in vitro* induced iron deficiency in peach rootstock, Mr.S 2/5. *In: XI Int. Sym. on Iron Nutr. and Interactions in Plant.* p. 116. June 23–28, University of Udine–Italy

Marschner, H., 1986. *Mineral Nutrition of Higher Plants*. Academic Press, Orlando, Florida, USA

Manthey, J.A. and D.E. Crowley, 1997. Leaf and root responses to iron deficiency in avocado. *J. Pl. Nut.*, 20: 683–93

Minotti, G. and S.D. Aust, 1987. The requirement for iron (III) in the initiation of lipid peroxidation by iron (II) and hydrogen Peroxide. *The J. Biol. Chem.*, 262: 1098–1104

Monterio, H.P. and C.C. Winterbourn, 1988. The superoxide–dependent transfer of iron from ferritin to transferring. *Biochem. J.*, 256: 923–8

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol. Plant.*, 15: 473–97

Nakano, Y. and A. Asada, 1981.Hydrogen peroxide is scavenged by ascorbate–specific peroxidase in spinach chloroplasts. *Plant Cell Physiol*., 22: 867–80

Noctor, G. and C.H. Foyer, 1998. Ascorbate and glutathione: keeping active oxygen under control. *Annu. Rev. Plant Physiol. Plant Mol. Biol*., 49: 249–79

Pinhero, R.G., M.V. Rao., G. Paliyath, D.P. Mur and R.A. Flercher, 1997. Changes in activities of antioxidant enzymes and their relationship to genetic and Paclobutrazol– induced chilling tolerant of maize seedling. *Plant Physiol*., 114: 695–704

Rao, M.V., G. Paliyath and D.P. Ormrod, 1996. Ultraviolet –B–and ozone–induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*. *Plant Physiol*., 110: 125–36

Shaw, B.P., 1995. Effects of mercury and cadmium on the activities of antioxidative enzymes in the seedlings of *Phaseolus aureus. Biol. Plant.*, 37: 587–96

Susin, S., J. Abian, F. Sanchez–Baeza, M.L. Peleato, A. Abadia, E. Gelpi and J. Abadia, 1993. Riboflavin 3 – and 5– sulfate, two novel flavins accumulating in the roots of iron–deficient sugar beet (*Beta vulgaris*). *J Biol. Chem.*, 268: 20958–65

Terry, N., 1980. Limiting factors in photosynthesis .1. Use of iron stress to control photochemical capacity *in vivo*. *Plant Physiol*., 65: 114–20

Tukendorf, A. and W.E. Rauser, 1990. Changes in glutathione and phytochelations in roots of maize seedling exposed to cadmium. *Pl. Sci*., 70: 155–66

Woodbury, W., A.K. Spencer and M.A. Stahman, 1971.An improved procedures using ferricyanide for detecting catalase isozymes. *Ann. Biochem.*, 44: 301–05

Zaharieva, T.B. and J. Abadia, 2003. Iron deficiency enhances the level of ascorbate, glutathione, and related enzymes in sugar beet roots. *Protoplasma*., 221: 269–75

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