CARYOLOGIA Vol. 60, no. 1-2: 96-101, 2007

Copper stress in *Cannabis sativa* roots: morphological and proteomic analysis

Bona Elisa[1], Francesco Marsano[1], Maria Cavaletto[1] and Graziella Berta[1*]

[1] Department of Environmental and Life Sciences, University of Piemonte Orientale "A. Avogadro", Via Bellini 25/G, 15100 Alessandria, Italy.

Abstract — This work investigated copper effect on the root morphology and root proteome of *Cannabis sativa* plants grown in the absence or in the presence of 150 ppm $CuSO_4$. Copper is an essential trace element in plants, but it becomes strongly phytotoxic at high concentrations. Root systems, though genetically determined, are very plastic and can be affected by a number of environmental factors, including metals. The resulting differences in root morphology and protein expression were indicative of a good *C. sativa* tolerance to copper. A group of proteins involved into plant adaptation to metal chronic stress has been detected.

Key words: *Cannabis sativa*, copper, hemp, proteome, root.

INTRODUCTION

Cannabis sativa is a multiple-use plant, widely employed in many types of non-food industries and providing raw material for the production of natural fiber, insulating board, rope, oil, varnish and paper. It is also a good candidate for soil phytoremediation as it is a tall plant with about 1-m deep roots that grow fast and easily in dense stands (Citterio *et al.* 2003). Hemp's ability to tolerate and accumulate heavy metals, such as lead, nickel, cadmium, zinc and chromium, is well documented (Linger *et al.* 2002; Kos and Les tan 2003; Kos *et al.* 2003; Citterio *et al.* 2003; Kos and Les tan 2004) with or without the use of chelates.

Root system morphology is involved in improving metal absorption, as a very branched root system is more efficient in exploiting soil than an elongated one, with a low number of laterals (Fitter *et al.* 1991). Root systems, though genetically determined (Berta *et al.* 1995), are very plastic and can be affected by a number of environmental factors, including metals (Berta *et al.* 1993; Chiatante *et al.* 2006). Furthermore, it has been shown that *C. sativa* is a tolerant organism that has evolved mechanisms allowing it to cope with high metal (Cd and Ni) concentration in soil (Citterio *et al.* 2005); it has been also shown that *C. sativa* tolerates, and in part accumulates, copper in the whole plant (Angelova *et al.* 2004).

Proteomic analysis is a new tool used to identify and characterize the entire set of proteins encoded by an organism. Several thousand individual proteins can be resolved by two-dimensional electrophoresis (2-DE) and identified by mass spectrometry; therefore, comparing 2-DE maps obtained under different biological conditions, it is possible to detect up- and down-regulated proteins (Cavaletto *et al.* 2004).

The aim of our work were to investigate in hemp i) its capability to accumulate copper; ii) the effects of this metal on root system morphology iii) the molecular effects of copper on the root proteome. In particular, we focused our attention on the differentially expressed proteins to better understand which proteins were simultaneously involved in the plant's defence system against copper.

MATERIALS AND METHODS

Plant material and growth conditions - Seeds of *Cannabis sativa* var. Felina 34 were washed with deionized water and germinated in controlled conditions (24°C, 16h day/8h night, 87.5 $\mu E \cdot m^{-2} \cdot s^{-1}$). Homogeneous seedlings were transplanted into individual pots containing 2 kg of a loam (Agral, Agrimont, Bolzano, Italy), quartz sand and gravel

* Corresponding author: phone: +390131360232; fax: +390131360390; e-mail berta@unipmn.it

mix (9:5:1). Five pots were added with a 150 ppm solution of $CuSO_4 \cdot 5\ H_2O$ and five were added with sterile water. Plants were grown in the controlled environment described above, and harvested 6 weeks after transplanting. All plants received weekly 400 ml of Long Ashton nutrient solution (TROTTA *et al.* 1996).

Measurement of leaf and root parameters - Photosystem II (PSII) activity was measured at 5 and 6 weeks of growth using a Handy-PEA (Hansatech Instruments, UK). The Fv/Fm (where Fv = Fm – Fo) ratio was used as a parameter to evaluate leaf stress induced by copper treatment, considering Fv/Fm values of 0.80-0.82 as normal values for unstressed plants (BJÖRKMAN and DEMMIG 1987), and lower values of Fv/Fm as an index of photosynthetic damage. Moreover, after harvesting, root and shoot fresh weight, stem length and diameter were measured; each sample was then stored at -80°C for proteomic analysis. Root and leaf morphometric analyses were performed with Win-RHIZO Pro V. 2002c (Regent Instruments, Quebec, Canada) (COSTA *et al.* 2002). Before analysis, roots were preserved in 70% ethanol. The following parameters were measured: total root length, total root surface area, total root projected area, total root volume, leaf area and number of tips.

Protein extraction, 2-DE and mass spectrometry- Proteins were extracted according to BESTEL-CORRE (BESTEL-CORRE 2002), with some modifications.

IEF was performed on IPG strips in an IPG-Phor unit (GE Healthcare Bio-Sciences) at 60 kVhs at 20°C on pre-cast 13-cm linear pH 3-10 strips. The second dimension was carried out with a Protean II xi system (Bio-Rad); 12% gels were run at 10°C under constant amperage (30 mA). Gels were Coomassie stained. The gels were scanned in a GS 710 densitometer (Bio-Rad). The

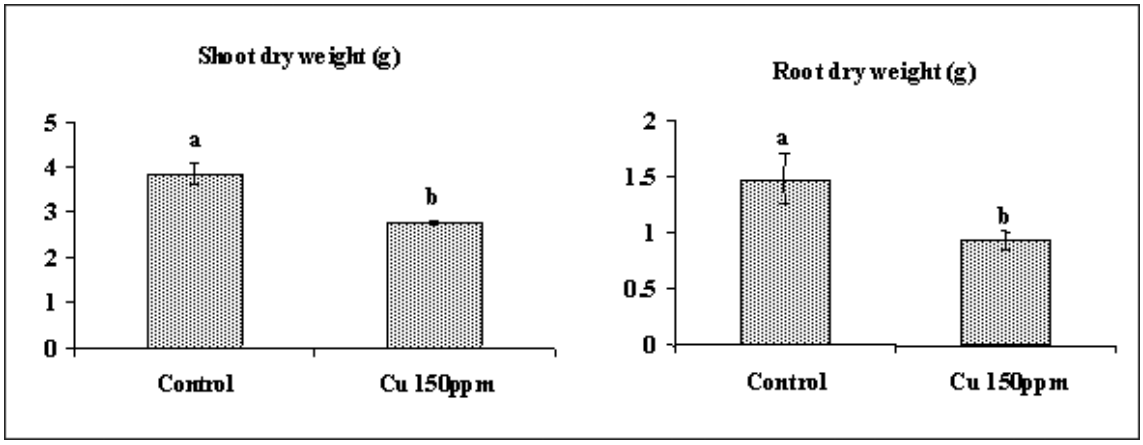

Fig. 1 — Shoot and root dry weight in control and in copper-treated hemp plants; a statistically significant reduction is shown in the latter.

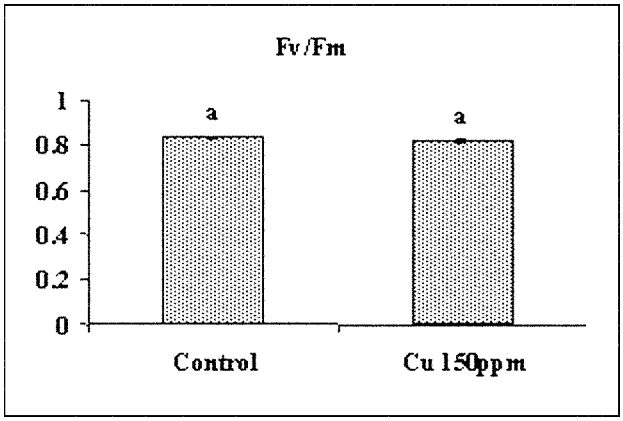

Fig. 2 — The graph show the photosynthetic activity data; Cu treated plants did not show evident symptoms of photosynthetic damage.

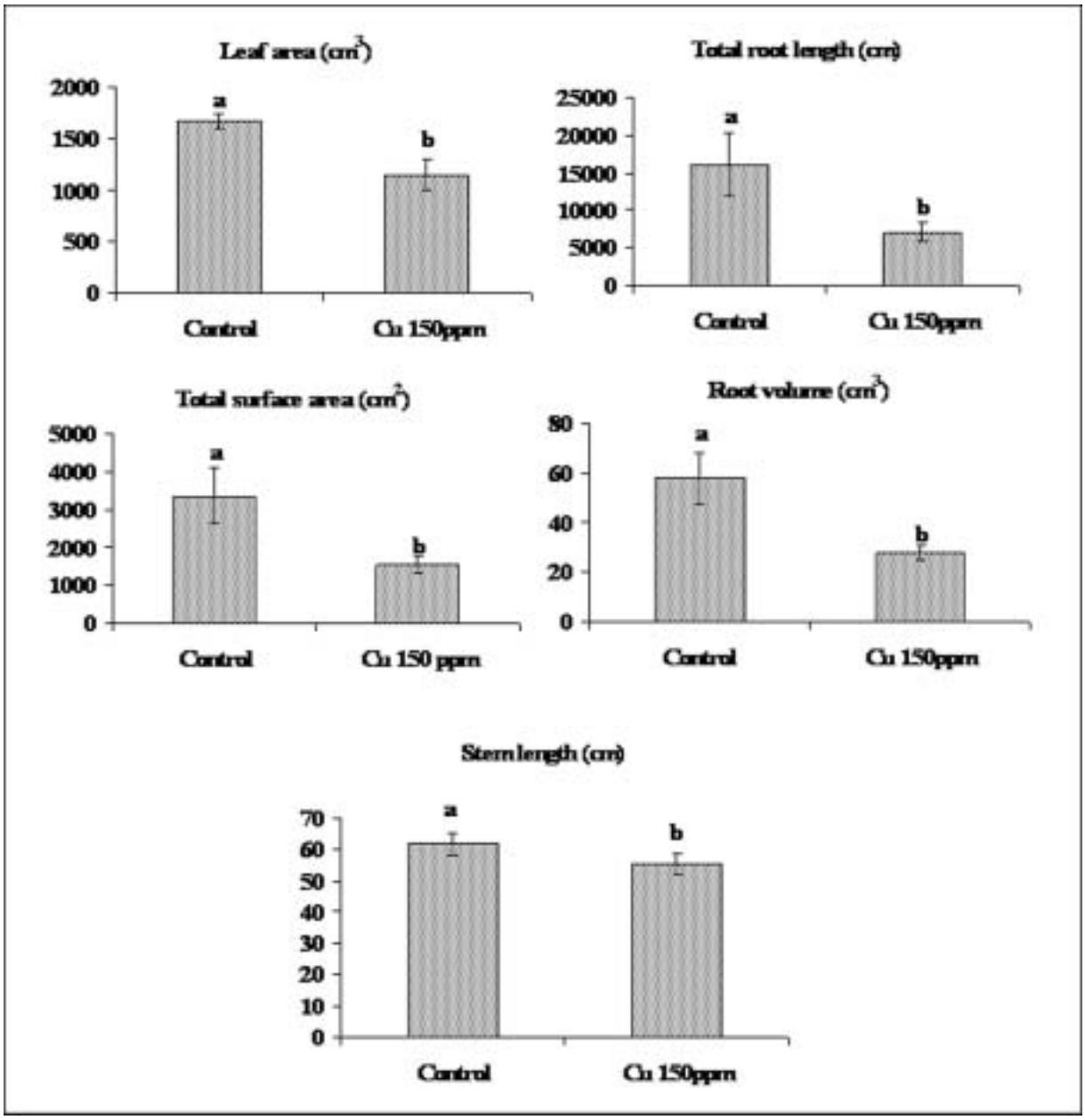

Fig. 3 — Root morphometric analysis. All considered parameters decrease significantly in copper-treated plants.

gel images were recorded and computationally analysed using PDQuest 7.3.1 (Bio-Rad). The differential expression analysis was performed comparing the quantity of matched spots in copper-treated gels (five replicates) versus the control gels (five replicates). We performed ANOVA using StatView 4.5 (Abacus Concepts, Berkeley, CA, USA) and $P = 0.05$ was adopted as the level of significance.

For MS analysis, spots of interest were cut from the gel and digested in-gel with trypsin (Roche, Segrate, Milano, Italy) as described by HELLMANN *et al.* (1995) MS/MS analysis of the digested peptide were performed according to BONA *et al.* (2007).

RESULTS AND DISCUSSION

Plant biomass and development - Copper stress induced a significant reduction in shoot and root dry weight (Fig. 1). In addition the aerial part of the plants (measured as leaf area and shoot length) was significantly reduced after copper

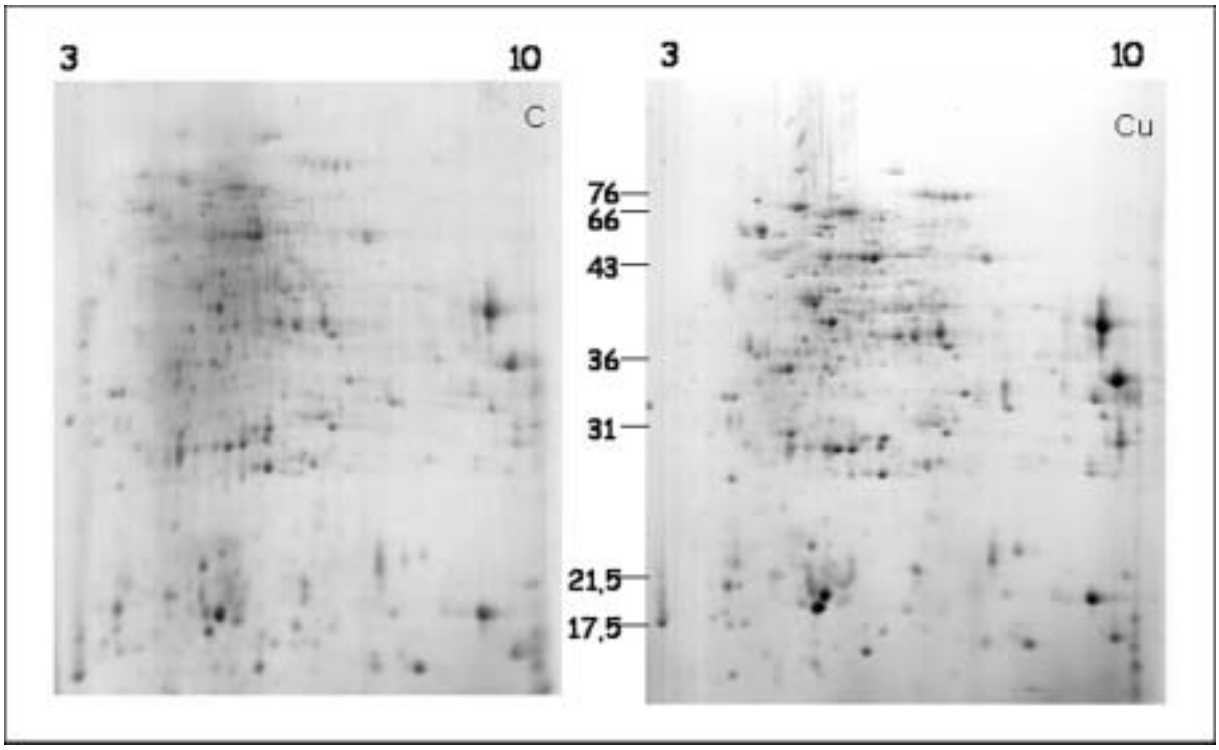

Fig. 4 — Two dimensional gel electrophoresis of *Cannabis sativa* root proteins stained with Coomassie Brilliant Blue G-250. The IEF was performed with 13 cm linear IPG strip pH 3-10, followed by SDS-PAGE on 12% gel. Molecular weight markers are shown.

treatment. Nevertheless treated plants did not show clear symptoms of chlorosis, and other analyses, carried out with the photosystem apparatus, showed no effect on PSII activity induced by copper at the concentration used here (Fig.2). All the root morphological parameters (total root length, mean root diameter, root surface area, and root volume) showed a significant reduction of the root system in response to copper treatment (Fig.3); root system morphology was also affected, as the degree of branching was significantly reduced in copper-treated plants (data not shown). A simpler, less branched root system as that induced by copper is less efficient in absorbing nutrients (Fitter 1991); thus, the copper effects result in a reduction of the absorbing root surface. A balance between cost-benefits related to root system development is also suggested.

Root proteome response to copper stress - Our approach was aimed at correlating morphological parameters and proteomic changes in the *C. sativa* root system. In figure 4, the 2-DE maps of control and copper-treated *C. sativa* root proteins are shown; about 300 spots were separated in a reproducible way. In order to highlight copper effects, an image analysis was performed on five replicates, and variations in spot area were confirmed by statistical analysis. Spots of interest were excised, trypsin-digested and identified by tandem mass spectrometry. As shown in table 1 copper treatment induced the disappearance of two proteins: thioredoxin-dependent peroxidase and 60S ribosomal protein L12; the down-regulation of seven proteins: enolase, cyclophilin, ABC transporter substrate-binding protein, glycine-rich RNA-binding protein, a putative peroxidase and elicitor-inducible protein EIG-J7, and the up-regulation of five proteins: aldo/keto reductase, a putative auxin-induced protein, 40S ribosomal protein S20, actin and mitochondrial formate dehydrogenase. Some of the identified proteins were typical stress proteins, e.g. thioredoxin-dependent peroxidase (Radyuk *et al.* 2003), the putative peroxidase (Lin and Kao 1998; Prazeres de Souza *et al.* 2002), cyclophilin and formate dehydrogenase, which is able to maintain a reducing environment (Hourton-Cabassa *et al.* 1998). Enolase is a multi-functional protein, it has been reported as responsive to many stresses, such as salt, cold and drought (Yan *et al.* 2005). The identification of ribosomal proteins and actin indi-

Table 1 — Differential expressed proteins. Proteins have been identified by proteomic analysis; since *Cannabis sativa* genome is unsequenced the reference organism for cross-species homology identification is shown.

Down regulated proteins	**M_r (kDa) / pI Theor**	**AC number (gi NCBI) and reference organism**
enolase	47.72 / 5.31	42521309 *Glycine max*
cyclophilin	18.15 / 8.95	*18076088* Ricinus communis
ABC transporter substrate-binding protein	38.39 / 8.38	27375524 *Bradyrhizobium japonicum*
Glycine rich RNA binding protein	16.89 /5.86	544424 *Arabidopsis thaliana*
Putative peroxidase	34.92 / 8.74	21536908 *Arabidopsis thaliana*
Elicitor inducible protein EIG-J7	20.34 / 5.07	40287496 *Capsicum annuum*
n.d.		
Thioredoxin-dependent peroxidase*	17.39/ 5.3	52851172 *Plantago major*
60S ribosomal protein L12*	17.71 / 8.8	40287508 *Capsicum annum*
Up regulated proteins	**M_r (kDa) / pI Theor**	**AC number (gi NCBI) and reference organism**
Aldo/keto reductase	37.47 / 5.7	2462763 *Arabidopsis thaliana*
Putative auxin induced protein	37.92 / 6.3	54290851 *Oryza sativa*
40S ribosomal protein S20	13.88 / 9.73	21542443 *Arabidopsis thaliana*
Actin	41.73 / 5.6	9082317 *Helianthus annus*
Mitochondrial formate dehydrogenase	42.02 /6.6	11991527 *Solanum tuberosum*

cated an involvement of the protein synthesis machinery and root architecture remodeling (Collins *et al.* 2000), respectively in response to copper stress. The up-regulation of aldo/keto reductase and the putative auxin-induced protein may be linked to their involvement in detoxification process by improving the scavenging capacity of the cell (Zhang and Riechers 2004; Zhang *et al.* 2005), and probably to their direct interaction with metal ions as 'copper chaperones' (Cecconi *et al.* 2002; Bona *et al.* 2007). Since cellular damage can occur both from an excess (copper is abl to promote the formation of reactive oxygen species) or a deficit of copper (it can act as cofactor in enzymatic antioxidant systems), a fine molecular control of free cellular copper levels is needed. All the proteins involved in the copper response may act to re-establish root system homeostasis, and may be considered as molecular markers for bioengineering new plants in phytoremediation projects.

REFERENCES

Angelova V., R. Ivanova, V. Delibaltova, Ivanov K., 2004 — *Bio-accumulation and distribution of heavy metals in fibre crops (flax, cotton and hemp).* Industrial Crops and Products, 19: 197-205.

Arru L., S. Rognoni, M. Baroncini, P. Medeghini Bonatti, Rerata P., 2004 — *Copper localization in Cannabis sativa L. grown in a copper-rich solution.* Euphytica, 140: 33-38.

Berta G., A. Fusconi, Trotta A., 1993 — *VA mycorrhizal infection and the morphology and function of root systems.* Environmental and Experimental Botany, 33: 159-173.

Berta G., A. Trotta, A. Fusconi, J.E. Hooker, M. Munro, D. Atkinson, M. Giovannetti, S. Morini, P. Fortuna, B. Tisserant, V. Gianinazzi-Pearson, Gianinazzi S., 1995 — *Arbuscular mycorrhizal induced changes to plant growth and root system morphology in Prunus cerasifera.* Tree Physiology, 15: 281-293.

Bestel-Corre G., E. Dumas-Gaudot, V. Poinsot, M. Dieu, J.F.Dierick, D. Tuinen, J. Remacle, V. Gianinazzi-Pearson, Gianinazzi S., 2002 — *Proteome analysis and identification of symbiosis-related proteins from Medicago truncatula Gaertn. by two-dimensional electrophoresis and mass spectrometry.* Electrophoresis, 23: 122-137.

Björkman O., Demmig B., 1987 — *Photon yeld of O_2 evolution and chlorophyll fluorescence characteristics at 77K among vascular plants of diverse origins.* Planta, 170: 489-504.

Bona E., F. Marsano, M. Cavaletto and Berta G., 2007 — *Proteomic characterization of copper stress response in Cannabis sativa roots.* Proteomics (In press).

Cavaletto M., M.G. Giuffrida, Conti A., 2004 — *The proteomic approach to the analysis of human milk fat globule membrane.* Clinical Chimica Acta, 347: 41-48.

Cecconi I., A. Scaloni, G. Rastelli, M. Moroni, P.G. Vilardo, L. Costantino, M. Cappiello, D. Garland, D. Carper, J.M. Petrash, A. Del Corso, Mura U., 2002 — *Oxidative modification of aldose reductase induced by copper ion.* The Journal of Biological Chemistry, 227: 42017-42027.

CHIATANTE D., A. DI IORIO, SCIPPA G.S., 2005 — *Root responses of Quercus ilex L. seedlings to drought and fire.* Plant Biosystems, 138: 124-139.

CITTERIO S., N. PRATO, P. FUMAGALLI, R. AINA, N. MASSA, A. SANTAGOSTINO, S. SGORBATI, BERTA G., 2005 — *The arbuscular mycorrizal fungus Glomus mosseae induces growth and metal accumulation changes in Cannabis sativa L.* Chemosphere, 59: 21-29.

CITTERIO S., A. SANTAGOSTINO, P. FUMAGALLI, N. PRATO, P. RANALLI, SGORBATI S., 2003 — *Heavy metal tolerance and accumulation of Cd, Cr and Ni by Cannabis sativa L.* Plant and Soil, 256: 243-252.

COLLINS D.A., N.S. ALLEN, IN: STAIGER C.J., BALUSKA F., VOLKMANN D., BARLOW P. 2000 — (Eds.), *Actin: A Dynamic Framework for Multiple Plant Cell Functions.* P. 145-163. Kluwer Academic, Dordrecht.

COSTA C., L.M. DWYER, X. ZHOU, P. DUTILLEUL, C. HAMEL, L.M. REID, SMITH D.L., 2002 — *Root development. Root morphology of contrasting maize genotypes.* Agronomy Journal, 94: 96-101.

FITTER A.H., 1991 — *Characteristics and Functions of Root Systems.* In: Waisel Y., Eshel A., Kafkafi U. (Eds), "Plant roots. The hidden half.", p. 3-24. Marcel Dekker, New York.

HELLMANN U., C. WERNSTEDT, J. GONEZ, HELDIN C. H., 1995 — *Improvement of an "in-gel" procedure for the micropreparation of internal protein fragments for amino acid sequencing.* Analytical Biochemistry, 224: 451–455.

HOURTON-CABASSA C., F. AMBARD-BRETTEVILLE, F. MOREAU, J. DAVY DE VIRVILLE, R. RÉMY, COLAS DES FRANCS-SMALL C., 1998 — *Stress induction of mitochondrial formate dehydrogenase in potato leaves.* Plant Physiology, 116: 627-635.

KHAN A.G., C. KUEK, T.M. CHAUDHRY, C.S. KHOO, HAYES W.J., 2000 — *Role of plants, mycorrhizae and phytochelators in heavy metal contaminated land remediation.* Chemosphere, 41: 197-207.

KOS B. AND LESTAN D., 2003 — *Influence of a biodegradable ([S,S]-EDDS) and nondegradable (EDTA) chelate and hydrogel modified soil water sorption capacity on Pb phytoextraction and leaching.* Plant and Soil, 253: 403-411.

KOS B. and LESTAN D., 2004 — *Soil washing of Pb, Zn and Cd using biodegradable chelator and permeable barriers and induced phytoextraction by Cannabis sativa.* Plant and Soil, 263: 43-51.

KOS B., H. GORC MAN, LES TAN D., 2003 — *Phytoextraction of lead, zinc and cadmium from soil by selected plants.* Plant Soil and Environment Journal, 49: 548-553.

KUNG, C.C.S., W.N. HUANG, Y.C. HUANG, YEH K.C., 2006 — *Proteomic survey of copper-binding proteins in Arabidopsis roots by immobilized metal affinity chromatography and mass spectrometry***.** Proteomics, 6: 2746-2758.

LIN J.N., KAO C.H., 1998 — *Effect of oxidative stress caused by hydrogen peroxide on senescence of rice leaves.* Botanical Bulletin of Academia Sinica, 39: 161-165.

LINGER P., J. MÜSSIG, H. FISCHER, KOBERT J., 2002 — *Industrial hemp (Cannabis sativa L.) growing on heavy metal contaminated soil: fibre quality and phytoremediation potential.* Industrial Crops and Products, 16: 33-42.

MANTOVI P., G. BONAZZI, E. MAESTRI, MARMIROLI N., 2003 — *Accumulation of copper and zinc from liquid manure in agricultural soils and crop plants.* Plant and Soil, 250: 249-257.

PRAZERES DE SOUZA I.R., V.M. CARVALHO ALVES, S.N. PARENTONI, A.C. DE OLIVEIRA, F.F. TEIXEIRA, J.W. MACADAM and PURCINO A.A.C., 2002 — *Change in root apical protein in response to aluminium in tolerant and sensitive maize inbred lines.* Brazilian Journal of Plant Physiology, 14(3): 219-224.

RADYUK S.N., SOHAL, R.S., ORR W.C., 2003 — *Thioredoxin peroxidases can foster cytoprotection or cell death in response to different stressors: over- and under- expression of thioredoxin peroxidase in Drosophila cells.* Biochemistry Journal, 371: 743-752.

ROUHIER N., JACQUOT J.P., 2005 — *The plant multigenic family of thiol peroxidases.* Free Radical Biology & Medicine, 38: 1413-1421.

TROTTA A., GC. VARESE, E. GNAVI, E. FUSCONI, S. SAMPO', BERTA G., 1996 — *Interaction between the soil-borne pathogen Phytophthora parasitica var. parasitica and the arbuscular mycorrhizal fungus Glomus mosseae in tomato plants.* Plant Soil, 185: 199-209.

YAN S., Z. TANG, W. SU, SUN W., 2005 — *Proteomic analysis of salt stress-responsive proteins in rice root.* Proteomics, 5: 235-244.

ZHANG D., L.K. TAI, L.L. WONG, L.L. CHIN, S.K. SETHI, KOAY E.S., 2005 — *Proteomic study reveals that proteins involved in metabolic and detoxification pathways are highly expressed in HER-Z/non positive breast cancer.* Molecular and Cellular Proteomics, 4: 1686-1696.

ZHANG Q., RIECHERS D.E., 2004 — *Proteomic characterization of herbicide safener-induced proteins in the coleoptile of Triticum tauschii seedlings.* Proteomics, 4: 2058-2071.

Received November 30th 2006; accepted January 30th 2007