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Copper toxicity in young maize (*Zea mays* L.) plants : effects on growth, mineral and chlorophyll contents, and enzyme activities

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Abstract

Changes in the growth parameters and in enzyme activities were studied in roots and leaves of 14-days old maize grown in a nutrient solution containing various copper concentrations (i.e. 0.01 to 10 μM). A significant decrease in root and leaf biomass was only found at 10 μM Cu. In contrast, changes in several enzyme activities occured at lower copper concentrations in the solution, corresponding to different threshold values which are lower than those observed for growth parameters. Peroxidase (POD) activity significantly increased in all investigated plant organs (i.e. 3rd-leaf, 4th-leaf and roots) in relation to their copper content. Additionally, glucose-6-phosphate dehydrogenase (G-6-PDH), and isocitrate dehydrogenase (ICDH) activities decreased in the leaves, especially in the 4th-leaf. However, the activity of malic enzyme (ME), G-6-PDH, glutamate dehydrogenase (GDH) and ICDH increased with the copper content in roots. According to the relationship between POD activity and copper content, the toxic critical value was set at 26 mg Cu per kg dry matter (DM) in roots and 21 mg Cu per kg DM in the 3rd-leaf. In roots, a new isoenzyme of peroxidase appeared for copper content above 12.6 mg Cu kg DM^{-1}. Measurement of enzyme activity, especially that of POD and Cu-specific changes in the (iso)peroxidase pattern, might be used as biomarkers to assess the phytotoxicity for maize grown on copper-contaminated substrata.

Introduction

When one deals with the diagnosis of a metal-contaminated soil, the ranking of intervention priorities, and the investigation of remediation techniques (including the follow-up on remediated soils), the fundamental question of 'how clean-is-clean' still remains. More research is needed to set a broad scale of guidelines which allows a better classification of soil quality (De Haan et al., 1989). Consequently, the No Observed Adverse Effect Concentration (NOAEC) approach was introduced (McGrath, 1993). It outlines that both the physico-chemical properties of a compound in the soil and the behavior of living organisms must be considered, in addition to the total trace element content in the soil. This necessitates the use of a battery of bioassays including various plants, organisms from soil biota, and animals.

Dose - response relationships used by agronomists for plants cultivated on metal polluted soils are mainly based on a decrease in yield, changes in mineral composition or increased contents of an undesirable compound such as some trace elements (Kabata-Pendias and Pendias, 1992; Lexmond, 1980; Macnicol and Beckett, 1985). However, there are numerous reports suggesting that the responses are much more complicated. A trace element is toxic to plants because it directly or indirectly affects the metabolic processes such as respiration, photosynthesis, CO_2-fixation, and gas exchange (Clijsters and Van Assche, 1985; Van Assche and Clijsters, 1990a; Vangronsveld and Clijsters, 1994), and other processes. As the metal concentrations in plant tissues rise, different mechanisms can be used to manage the stress before the sudden

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onset of chronic toxicity (Macnair and Baker, 1994). Physiological and metabolic biomarkers are often early indicators of stress and can add information about the degree of chemical exposure at the organism level (Cormier and Daniel, 1994; Ernst and Peterson, 1994; Vangronsveld et al., 1996). Changes in plant metabolism can be a response to dose and exposure that could be used to assess the classification of soil quality. Such an approach was first developed for Zn, Cd and Cu toxicity for beans (*Phaseolus vulgaris*) (Van Assche and Clijsters, 1987; 1990b; Vangronsveld and Clijsters, 1992). However, different species grown in exactly the same soil may be affected differently by trace elements. Among cultivated plants, plant species such as maize (*Zea mays*) were found to be moderately tolerant to Cu as compared with sensitive ones such as tomato (*Lycopersicon esculentum*) (Adriano, 1986).

In France, large Cu burden in soil has occured especially in old vineyards and orchards that may lead to toxicity in subsequent crops (Delas, 1963). Although field-based verification of biomarker responses is needed, in particular under Cu stress, primarily laboratory-based work is essential for developing biomarkers. Changes in the morphology and ultrastructure of maize roots under Cu stress have been described (Ouzounidou et al., 1995). It is well known that roots accumulate higher amounts of Cu than the aerial parts (Cathala and Salsac, 1976; Lepp, 1981; Lexmond and van der Vorm, 1981). At the cellular level, Cu chelation and compartmentation (e.g. electron-dense Cu-containing deposits) within the vacuole protect cell function under stress conditions (Lidon and Henriques, 1994; Meharg, 1993). The production of intracellular metal-binding compounds (metallothioneins and/or phytochelatins) increased as Cu was accumulated in roots (Lidon and Henriques, 1994). Some enzymes have been proposed as biomarkers of the overall response of plants (beans) to metal exposure. When plants accumulate toxic amounts of metals such as Zn, Cu, Cd or Pb, the activity of enzymes (e.g. peroxidases, malic enzyme, isocitrate dehydrogenase,...) increases in leaves and/or in roots (for a review see Vangronsveld and Clijsters, 1994). In lettuce (*Lactuca sativa*) and bean seedlings, in the whole plant and the primary leaves respectively, under Cu stress growth is inversely proportional to the induction of peroxidase activity (Mukherji and Das Gupta, 1972; Van Assche and Clijsters, 1987).

For maize, data on changes in metabolic processes as a result of increased Cu concentrations in tissues, before plants reveal a decrease in growth, are scarce. In the present paper, the effect of Cu on enzyme activities involved in energy metabolism, i.e. malic enzyme (ME), glucose-6-phosphate dehydrogenase (G-6-PDH), glutamate dehydrogenase (GDH) and isocitrate dehydrogenase (ICDH), and in the detoxification of reactive oxygen forms, i.e. (guaiacol)-peroxidase (POD), together with morphological parameters was investigated in maize roots and leaves to get more information about the nature of the in vivo effects of Cu, and Cu-specific changes in the (iso)peroxidase pattern. Other candidates (e.g. superoxide dismutases, catalases) (Vangronsveld and Clijsters, 1994) would be further investigated. The biological effects were compared with data of chemical plant analysis for setting up acting toxic threshold values for Cu concentration in the leaves and roots of maize plants at the fully developed 4th-leaves growth state.

Materials and methods

Plants

Zea mays L. cv. Volga seeds were rinsed three times in distilled water and further surface sterilized by immersion for 20 min in hydrogen peroxide 3% vol/vol. Thereafter, the seeds were rinsed twice with distilled water, then aseptically transferred into a pyrex dish and displayed on a filter paper sheet imbibed with 2m*M* $CaSO_4$. A plastic film, with some small holes facilitating gas exchanges but reducing water loss, was used to seal the dish. Germination took place in darkness at 25°C in an incubator. After 3 days, eight uniform seedlings were transferred to 2 L, 20-cm-high polyethylene jars wrapped in a black plastic film, previously rinsed with nitric acid 2 *N* and distilled water. They contained 1.5 L of a modified Hoagland nutrient solution (HNS) (Ewitt, 1966). Cu was not added to the HSN during the preculture phase. To reduce Cu complexation, ferrous sulfate was used instead of a chelator such as ethylene-diamine-tetraacetic acid (EDTA). The nutrient solution was aerated continuously and renewed every 2 days. Growth conditions were : 25/20 °C day/night temperature; 75% relative humidity; 16/8 h photoperiod; 400 μmol m^{-2} s^{-1} photosynthetic photon flux density at leaf level. After 3 days, plant cultivation was performed with the same device and growth conditions but pentahydrated $CuSO_4$ was added to the HSN at the following concentrations: 0.01, 0.05, 0.1, 0.2, 0.5, 1, 2, 3, 5, 7, and 10 μM Cu. Fourteen days

after sowing, five uniformly sized plants were collected. Cu levels in the HSN and culture duration were choosen as a result of preliminary investigations. They showed that in these conditions visible symptoms of Cu toxicity occured on both roots and aerial plant parts at the highest Cu concentration, and that plants reached the 4-leaves stage level.

Just before harvest, several morphological parameters were measured for characterizing the plant growth: i.e. shoot length (from the nodal root insertion on the stem to the tip of the longest leaf), length and width at mid-length of each leaf. Leaf area was calculated from experimental data using the following equation, $Y=0.8077 \times Lf \times Lw + 0.3044$ (Lf-leaf length, Lw-leaf width at mid-length) which was previously established on maize plantlets at the 4th-leaf growth level using the surface integration method. Chlorophyll was measured. Two discs (0.8 cm diameter) leading to a 1 cm^2 leaf area were collected at the mid-length of the leaf, from both mid-rib sides. Discs were immersed in 3 mL *N,N*-dimethylformamide (DMF) kept at 4 °C. After incubation at 4 °C for 48 h, chlorophyll a (Chla), chlorophyll b (Chlb) and total carotenoid amounts were determined by UV-Vis spectrophotometry (Varian Cary 1E). The absorbance of the solution was measured at 470, 647 and 664.5 nm. Formulae and extinction coefficients used for determination of chlorophyllous pigments were published by Lichtenhaler and Wellburn (1983), and Blanke (1990).

Each plant was divided into four plant parts : roots, 3rd-leaf (L3), 4th-leaf (L4), and the remaining shoots. The fresh weight (FW) of all plant parts was determined. They were further rinsed in a large volume of distilled water. For each treatment, L3, L4 and roots samples were pooled and then cut with ceramic scissors. The resulting small pieces were sampled (1 g FW) twice and immediatly frozen in liquid nitrogen for enzyme measurements (storage at a temperature of -80 °C). The remaining ones were weighed (FW) and oven-dried at 80 °C to constant weight for determining dry matter (DM) and mineral composition in plant samples.

Enzyme assays

Frozen material (1 g FW) was homogenized with an ice-cooled mortar and pestle in 5 mL of 0.1 *M* tris-HCl buffer pH 7.8, containing 1 m*M* dithiotreitol and 1 m*M* EDTA. The homogenate was squeezed through a nylon mesh and centrifuged at 12000 *g* and 4 °C for 10 min. The supernatant was collected, and on this crude extract, the activity of the following enzymes was measured spectrophotometrically with a Varian Cary 1E as previously described by Van Assche et al. (1988), and Van Assche and Clijsters (1990b) : peroxidase (POD; E.C.: 1.11.1.7), malic enzyme (ME; E.C.: 1.1.1.40), glucose-6-phosphate dehydrogenase (G-6-PDH; E.C.: 1.1.1.49), glutamate dehydrogenase (GDH; E.C.: 1.4.1.2) and isocitrate dehydrogenase (ICDH; E.C.: 1.1.1.42). In particular, guaiacol was used for determining total POD activity. The enzyme activity was expressed in mU per mg soluble protein. Soluble protein content in both leaf and root extracts was determined using the Bio-Rad protein assay according to Bradford (1976) with bovine serum albumin as standard.

Anionic (iso)peroxidases were separated by polyacrylamide gel electrophoresis on a 7.5/20% gradient slab gel, and stained enzymatically with 0.04% benzidine and 0.006% H_2O_2 for 1.5 h at 37 °C (Van Assche and Clijsters, 1990b). The gels were scanned densitometrically at 632 nm.

Plant samples analysis

Oven dried plant samples were wet-digested in nitric acid and hydrogen peroxide (Mench et al., 1994). Cu concentration in digested solutions was determined by air-acetylene flame atomic absorption spectrophotometry (Varian spectra A 20). P, K, Ca, Mg, Fe, Mn, and Zn concentrations were measured by inductively coupled plasma emission (Varian liberty 200). Quality control for plant analysis was performed by analyzing blanks and certified reference material (ryegrass BCR 281, Community Bureau of Reference, Commission of the European Communities) using the same method in triplicate. For both standard and samples, three measurements were done with a standard deviation less than 2%.

Data processing

The best function through the sets of x,y data was calculated with a curve-fitting programme (Fig. P, Biosoft, Ferguson, USA) containing linear, quadratic, polynomial, exponential and logarithmic functions. The threshold value for phytotoxicity of Cu was defined as the concentration in the plant tissue above which growth was reduced or metabolism changed. It was calculated from the intersection of the two straight lines, obtained after plotting the logarithm of the studied parameter (e.g. root yield or enzyme activity) against

the reciprocal of Cu content in plant tissue (Van Assche et al., 1988). The two linear functions were optimized by two-phase linear regressions.

Correlations between variables were calculated with STATITCF 4.0 (ITCF, Boigneville, F). Data for the morphological parameters were analyzed using a conventional analysis of variance, and the Newman-Keuls test at the 5% level.

Results

Visual symptoms of toxicity

No visible toxicity symptoms appeared when maize was grown in HNS with Cu concentration ranging from 0.01 to 5 μM. However, plants grown in HNS with 7 and 10 μM Cu exhibited a chlorosis. The lower half of L3 and L4 leaves showed a yellow coloration along the length of the leaf (darker green mid-rib constrasting against a yellow background). Moreover, the midribs at the underside of the leaves became purple as previously described by Lexmond and van der Vorm (1981). At 10 μM, roots were less branched, thus indicating that secondary and tertiary roots were more sensitive to excess Cu.

Cu content of plant parts and growth

Maize plants grown for 14 days in Cu concentrations ranging from 0.01 to 10 μM showed an increase in Cu concentrations for all plant organs examined (Figure 1). In roots Cu ranged from 3.2 mg to 187 mg kg DM^{-1} whereas Cu in leaves increased from 3.4 mg to 62 mg kg DM^{-1} in L3 and from 1.6 mg to 30 mg kg DM^{-1} in L4. Cu content in plant organs increased curvilinearly with Cu in the nutrient solution. The curve plots were best fitted according to the classical Michaelis-Menten function [$Y = Cm \times X/(Km+X)$], with Cm = maximum concentration (mg Cu kg DM^{-1}) and Km = affinity coefficient (μM); the following parameters were estimated : Cu in root, Km = 49.9, Cm = 1135 (r^2 = 0.99); Cu in L3, Km = 2.8, Cm = 78.9 (r^2 = 0.96); Cu in L4, Km = 1.6, Cm = 37.8 (r^2 = 0.91).

In L3 the amount of Cu was also calculated in relation to the leaf area. Mean values ranged from 3.9 up to 166 ng Cu cm^{-2} . The plot also can be fitted by a simple Michaelis-Menten equation (Km = 2.4 μM, maximum density = 195 ng Cu cm^{-2}, r^2 = 0.93), however the data distribution may suggest a two-component mechanism (Figure 2).

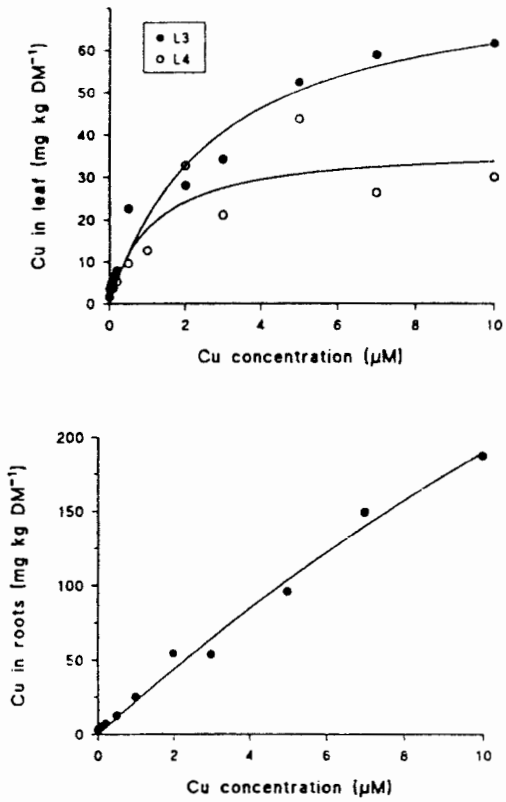

Figure 1. Cu concentrations (mg kg DM^{-1}) in the 3rd leaf (L3), the 4th leaf (L4), and in roots of *Zea mays* L. cv. Volga after two weeks of growth in a modified Hoagland nutrient solution with increasing Cu concentrations.

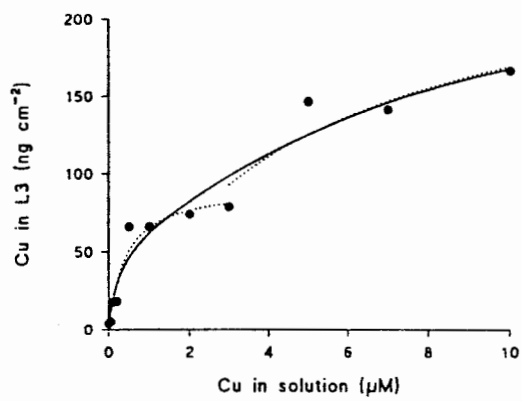

Figure 2. Changes in Cu accumulated per leaf area in the 3rd leaf of *Zea mays* L. cv. Volga after two weeks of growth in a modified Hoagland nutrient solution with increasing Cu concentrations.

Plants exposed to Cu in the concentration range 0.01-7 μM did not show any change in morphological parameters, i.e. root and leaf yield, height, leaf area. Shoot length plotted against Cu in HNS is shown in Figure 3a as an example. The effects of Cu on these parameters became evident at 10 μM when Cu content

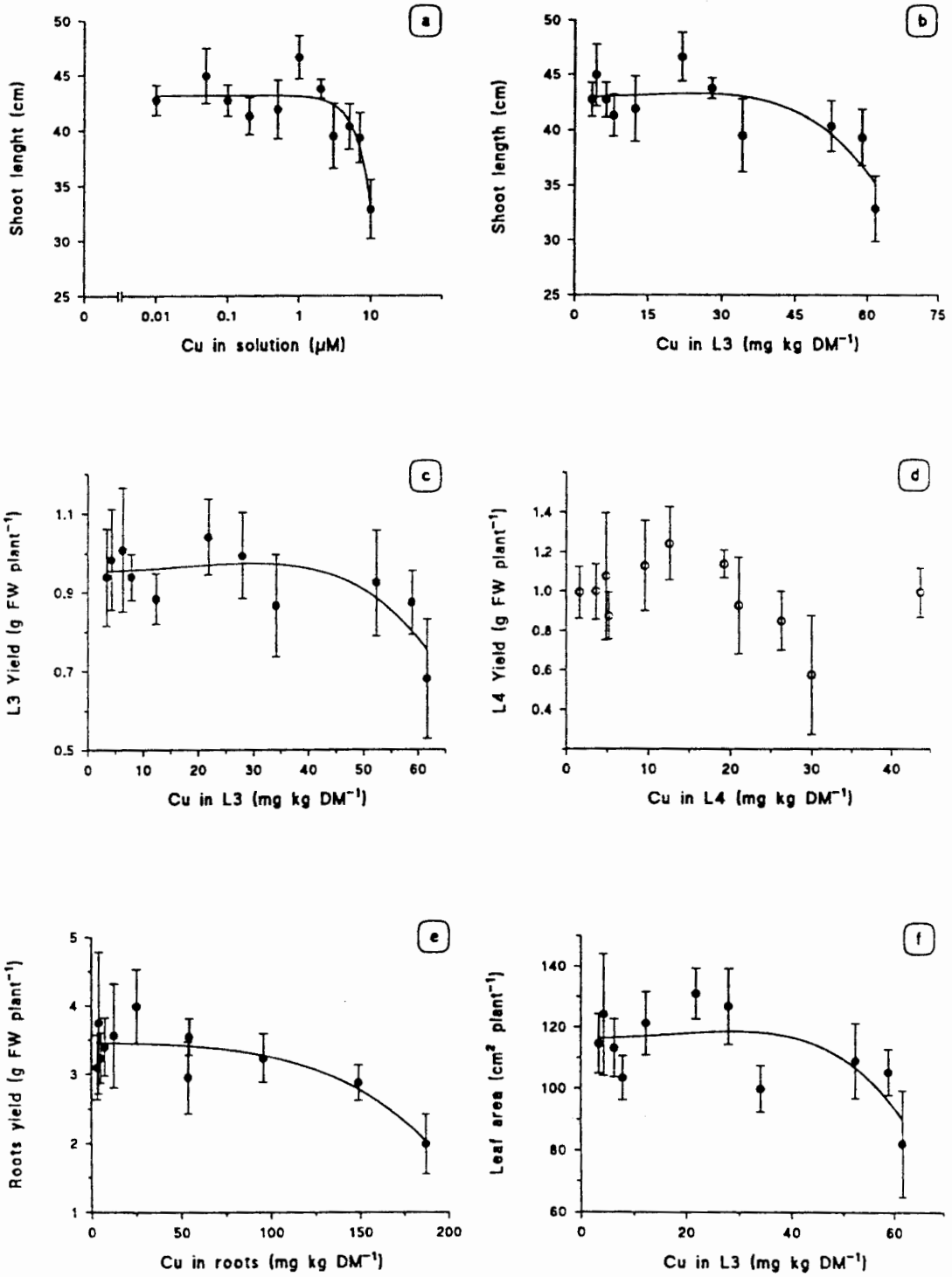

Figure 3. Changes in morphological parameters, i.e. (a, b) shoot length, (c) 3rd (L3) and (d) 4th (L4) leaf yields, (e) roots yield, (f) total leaf area of *Zea mays* L. cv. Volga in relation to Cu concentrations either in a modified Hoagland nutrient solution or in plant parts.

in organs reached 187 mg kg DM^{-1} in roots and 62 mg kg DM^{-1} in L3 (Figures 3b–3f). Although, the mean values for plants grown at 10 μM Cu were numerically lower than those for seedlings cultivated at lower Cu concentrations, the decrease was not statistically significant ($p < 0.05$). In contrast, extraction of L4 with DMF showed a very early decrease in chlorophyll (Figures 4a–4c); a sharp decrease occured in Chl a and b and in total carotenoids, especially when Cu concentration in L4 exceeded 13 mg Cu kg DM^{-1}. In L3, the photosynthetic pigment concentration was not affected by Cu (Figures 4a–4c).

Enzyme activities

First, total soluble protein content was examined in leaf and root samples. In L3, this parameter ranged from 9 to 12 mg g FW^{-1} but did not vary significantly in relation to the Cu content of leaf L3. Protein content appeared to be numerically higher in L4 (13.5 mg g FW^{-1}) when the Cu content of this leaf was below 9 mg kg DM^{-1} ; above this value, it decreased to 10 mg g FW^{-1} for all treatments. In roots, total soluble protein content increased slightly from 3 to 3.8 mg g FW^{-1} as Cu content increased.

The activity of the following enzymes was significantly correlated with leaf Cu content : i.e. POD (0.92**) and G-6-PDH (-0.47*) in L3, and POD (0.73**), ICDH (-0.71**), and G-6-PDH (-0.68**) in L4 (* and ** : significant at 1% and 5%, respectively). POD activity in leaves rose in relation to the increase in leaf Cu content (Figures 5a and 5b). In contrast, the activities of the NAD(P)H reducing enzymes such as ICDH and G-6-PDH decreased, especially in L4 (Figures 5d and 5f). In roots, the activity of all the enzymes measured was significantly correlated with the Cu content (e.g. POD : 0.79**, ICDH : 0.75**). POD activity in roots increased as in the leaves (Figure 6a). However, the behaviour of NAD(P)H enzymes was different compared to the leaves, because their activity increased with the root Cu content (Figures 6c-e). For every enzyme showing a correlation with Cu content in plant tissue, a quadratic function fitted best with the experimental data (Figures 5 and 6).

Toxic threshold values for both leaf and root Cu contents, above which enzyme activity either increased or decreased were calculated as described above. Figure 7 shows these threshold values for POD activity in maize roots and leaves as an example. The values obtained are presented in Table 1. Apparently, POD activity was stimulated when the leaf Cu content exceeded 21 mg in L3 and 7 mg in L4 per kg DM. In L4 threshold values for Cu toxicity were similar when calculated from the data of G-6-PDH or chloroplastic

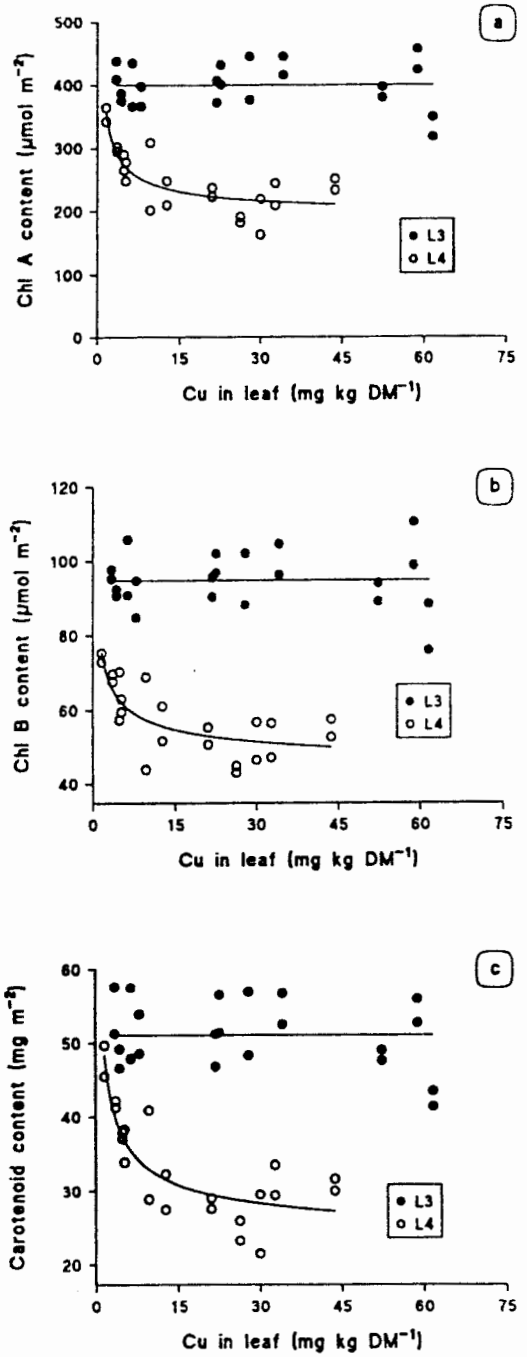

Figure 4. Changes in (a-c) chlorophyllous pigment content for the 3rd (L3) and the 4th (L4) leaves of *Zea mays* L. cv. Volga in relation to Cu concentrations in the respective leaf tissue, after two weeks of growth in a modified Hoagland nutrient solution.

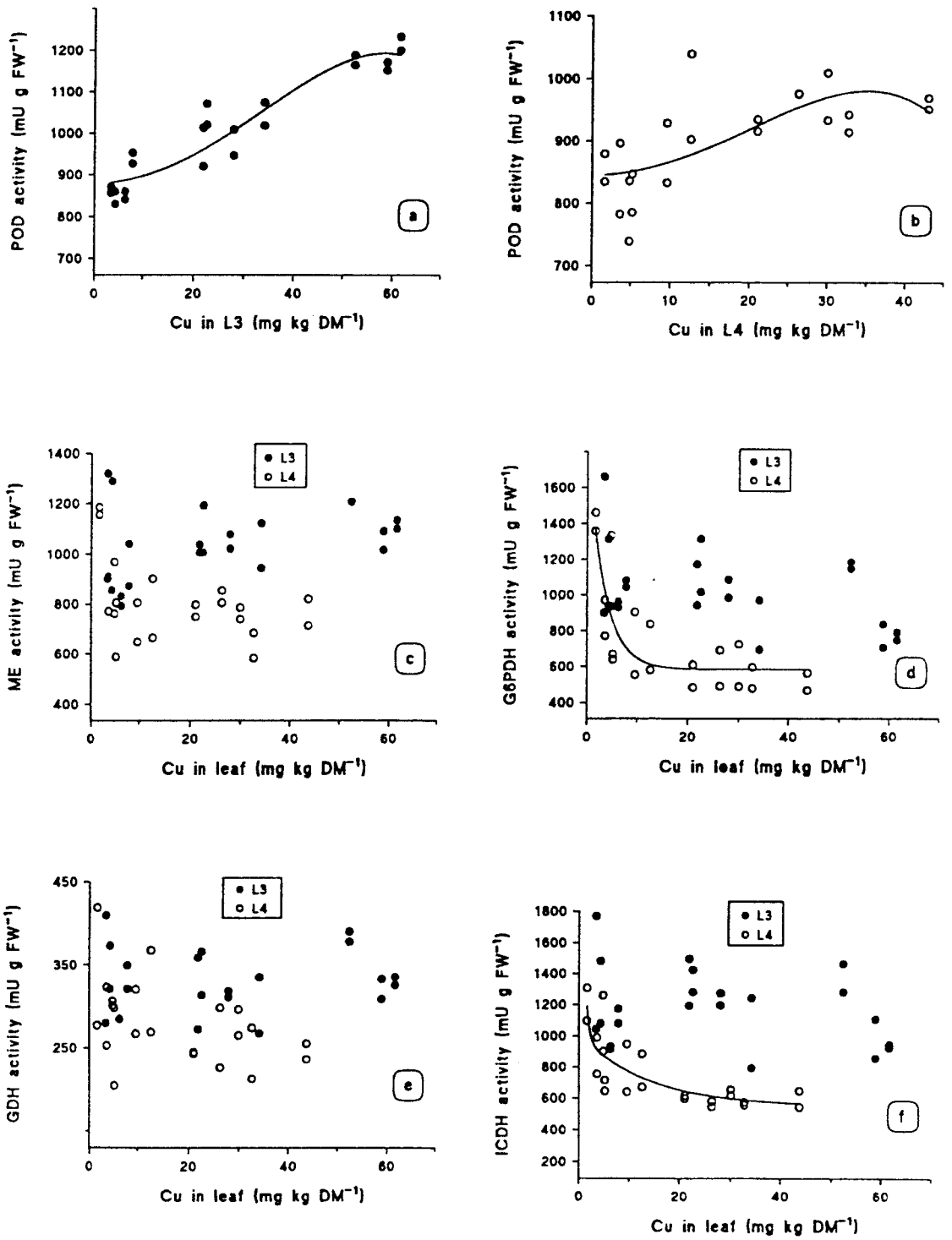

Figure 5. Enzyme activity in the leaf extracts of *Zea mays* L. cv. Volga as a function of Cu concentrations in the 3rd leaf (L3) and the 4th leaf (L4): (*a*) (guaiacol)-peroxidase in L3, (*b*) (guaiacol)-peroxidase in L4, (*c*) malic enzyme, (*d*) glucose-6-phosphate dehydrogenase, (*e*) glutamate dehydrogenase, and (*f*) isocitrate dehydrogenase.

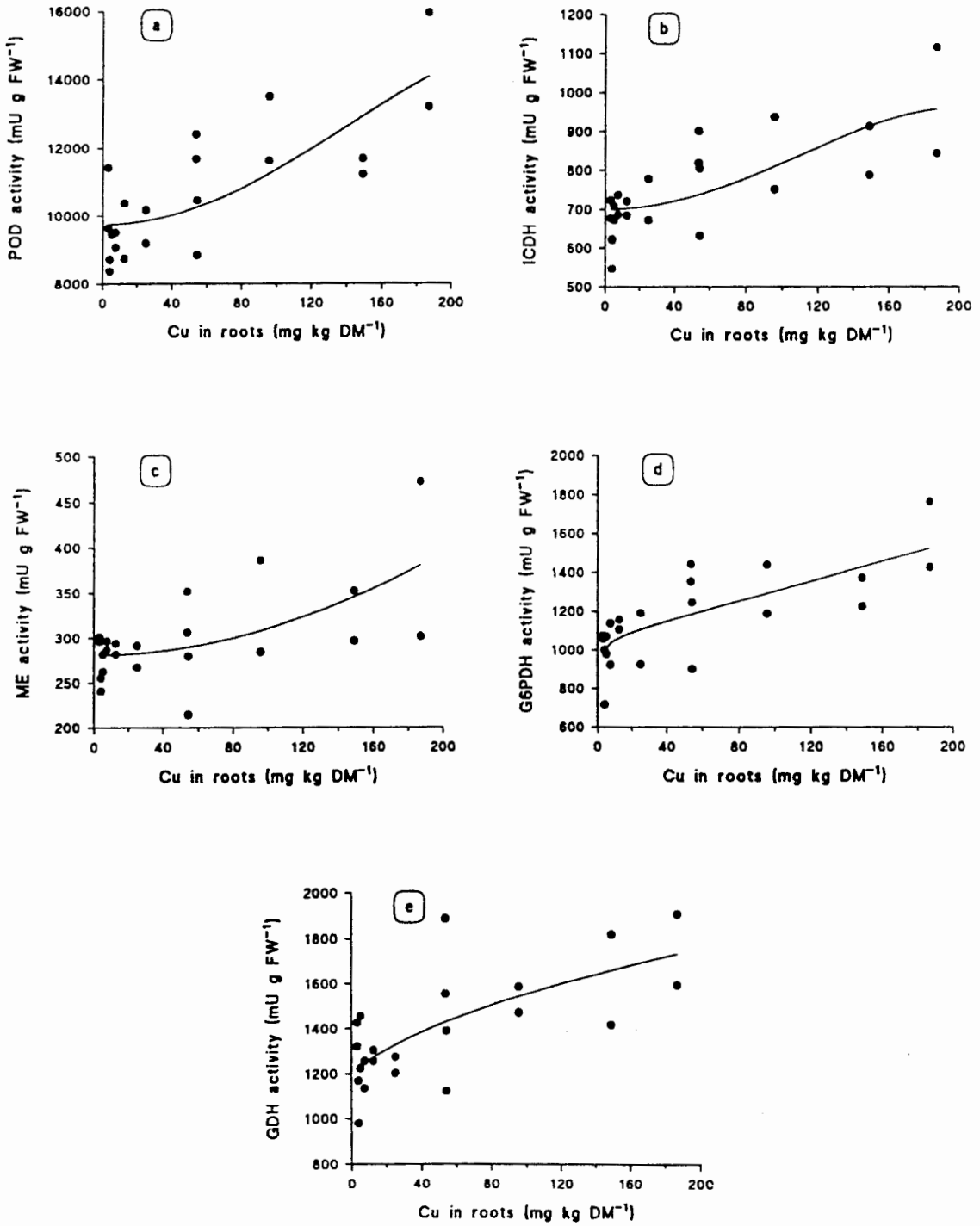

Figure 6. Enzyme activity in the root extracts of *Zea mays* L. cv. Volga as a function of root Cu concentrations: (*a*) (guaiacol)-peroxidase, (*b*) isocitrate dehydrogenase, (*c*) malic enzyme, (*d*) glucose-6-phosphate dehydrogenase, and (*e*) glutamate dehydrogenase.

pigments. In roots, a threshold value of 26 mg Cu per kg DM is proposed according to the results of POD activity. This value corresponds with the data obtained for the other enzymes measured in roots (ICDH, G-6-PDH, ME and GDH).

That Cu toxicity is reflected by the POD activity in the roots is confirmed by the changes in (iso)peroxidase pattern. The gels show that a specific (iso)peroxidase was induced by Cu in roots when its concentration exceeded 0.5 μM in the HNS, corresponding to 12.6 mg Cu kg DM^{-1} in tissue (Figure 8). The supplementary band (1) is more intense for 1 μM or higher concentrations; thus it was present in all root samples containing more Cu than the threshold value of 26 mg Cu kg DM^{-1}. In contrast, no change in isoenzyme pattern was noticed for peroxidase in both leaves L3 and L4.

Mineral composition of plant tissue

Mean concentration for all treatments, standard deviation, and significant Cu effect on element content in organs are listed in Table 2 for each element studied. No changes occured in maize roots for P, K, Ca, Mg, Fe, Mn, and Zn. In contrast, Ca and Mg concentrations increased in L3 with the Cu content of that organ. Ca and Mg concentrations in the L3 of plants grown in 10 μM Cu compared with their respective mean values calculated for plants having a Cu concentration below the Cu threshold value (21 mg kg DM^{-1}) revealed a maximum increase by 52% and 31% for Ca and Mg. Similar relationships, but in a lesser extent, were found in L4. P content in L3 slightly decreased as Cu content increased.

Discussion

Toxic threshold values in plant tissue can be used for rapid investigation of the soil quality regarding the food chain, for the follow-up of the remediation of metal polluted soils, and for a critical evaluation of the effectiveness of soil treatments. Two classical ways have been generally used by the agronomists for establishing these values. When the trace elements investigated are primarily phytotoxic (e.g. Zn, Ni and Cu) critical values for toxicity were calculated from the relationship between plant element concentration and dry matter yield (e.g. decrease at 10% or 25% level for above ground plant parts or root biomass) and/or root elongation and growth (Keddy et al., 1994; Macnicol

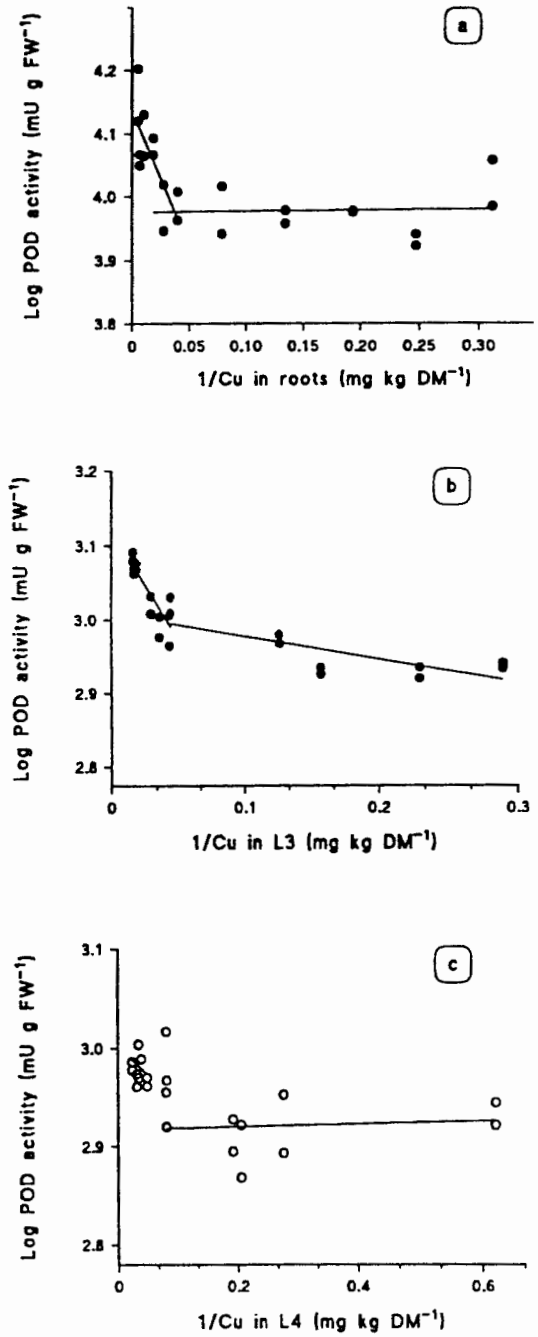

Figure 7. Decimal logarithm of peroxidase activity in tissue extracts plotted against the reciprocal of Cu concentration in the plant tissue for (a) the roots, (b) the 3rd (L3) leaf, and (c) the 4th (L4) leaf of *Zea mays* L. cv. Volga, after two weeks of growth in a modified Hoagland nutrient solution.

and Beckett, 1985). For trace elements which are primarily toxic towards animals and humans such as Hg, Cd, Tl and Pb, toxic threshold values were deducted

Table 1. Toxic threshold values (mg Cu kg DM^{-1}) calculated from the functions fitting the relationship between either growth parameter or enzyme activity and Cu content in the respective tissues of *Zea mays* L.

	Plant organs		
	Roots	L3	L4
Enzyme activity			
POD	26.0	21.4	7.2
ICDH	25.5	ns	20
G-6-PDH	28.4	54	10
ME	25.0	ns	ns
GDH	25.6	ns	ns
Growth parameters			
Shoot length	46	27	14
Leaf area	89	27	ns
Leaf yield		52	ns
Root yield	92		
Chlorophyllous pigments			
Chlorophyll a		ns	13.2
Chlorophyll b		ns	10.6
Total carotenoids		ns	15.4

ns: not significant; for all determined threshold value, the correlation coefficient (r) of the functions fitting was either $r < 0.1$ or $r > 0.9$.

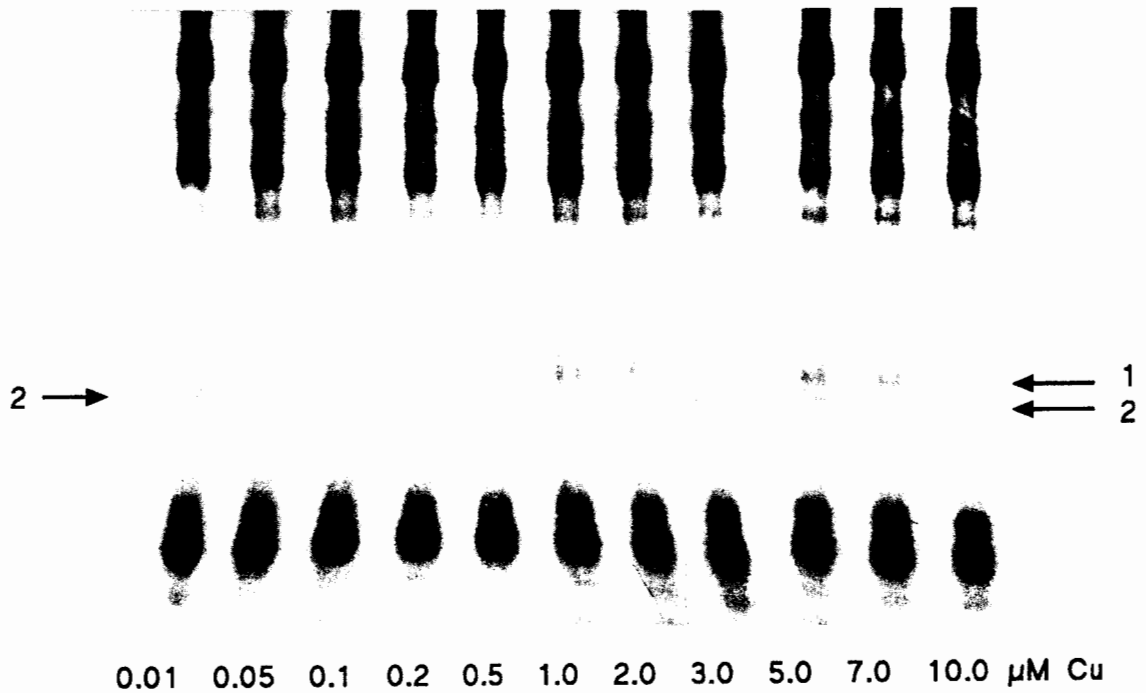

Figure 8. Anionic (iso)peroxidase pattern in maize roots protein extracts separated by polyacrylamide gel electrophoresis and stained enzymatically with 0.04% benzidine and 0.006% H_2O_2 ; *Zea mays* L. cv. Volga were cultivated two weeks in a modified Hoagland nutrient solution (HNS) with increasing Cu concentrations from 0.01 μM to 10 μM. Noticed the (iso)peroxidase band 1 when Cu concentration set at 0.5 μM in HNS, and above this value as well.

Table 2. Mineral concentrations (mg kg DM^{-1}) in plant organs for maize (*Zea mays* L. cv. Volga) cultivated in a modified Hoagland nutrient solution with Cu concentration ranging from 0.01 to 10 μM: mean and standard deviation values calculated for all Cu treatments, correlation with Cu concentration in the respective plant organs, and Cu effect on element concentration (●: negative interaction ; ○: additive interaction; ns: no significant effect)

	Plant organs								
	Roots			L3			L4		
	Mean	SD	Cu effect	mean	SD	Cu effect	mean	SD	Cu effect
P	15744	± 3500	ns	30234	± 2550*	●	22849	± 2320	ns
K	57236	± 2918	ns	61610	± 2594	ns	57742	± 4656	ns
Ca	1226	± 308	ns	2866	± 513**	○	1549	± 408**	○
Mg	1099	± 248	ns	1618	± 224**	○	1485	± 204**	○
Fe	4296	± 1219	ns	104	± 14	ns	85	± 13*	○
Mn	6	± 2	ns	17	± 4	ns	18	± 4*	○
Zn	19	± 10	ns	26	± 18	ns	24	± 9	ns

*Significantly correlated at 95% confidence level. **Significantly correlated at 99% confidence level.

from allowed dietary daily intake (Ewers, 1991). Up to now, the molecules involved in tolerance mechanisms are scarcely used for establishing the toxic threshold value for trace elements in plant. The relationships were mostly established at metal concentrations in the solution such as 10 or 100 mM which are caricatural compared generally to the soil solution (Vangronsveld and Clijsters, 1994). At these concentrations, both root and leaf dry-matter yields are already damaged.

In the present investigation, a Cu concentration range was applied which induced a slight numerical decrease of the dry matter yield of maize without real significant effects on morphological parameters. Among the enzymes investigated, POD showed a dose-response relationship between enzyme activity expressed per g tissue FW and tissue Cu concentration for all plant organs investigated (i.e. L3, L4 and roots), although effects on dry-matter yields were not significant. The increase in POD activity is likely to be related to oxidative reactions corresponding to an increase in peroxides and free radicals in the plant cells. No doubt that causes are multiple (Van Assche and Clijsters, 1990a). Activation of oxygen has been proposed as a general reaction on several stress factors including metals, and the very reactive intermediary forms of oxygen could be quenched by the induction of specific enzymes such as POD, superoxide dismutase (SOD) and catalase (Elstner et al., 1988). Cu-mediated free radical formation leading to lipid peroxidation has been demonstrated in isolated chloroplasts (Sandmann and Böger, 1980) and intact roots (De Vos et al., 1993). These radicals initiate lipid peroxidation and finally destabilize the thylakoïd membrane (Van Assche and Clijsters, 1990a; Clijsters et al., 1991). Disintegration of biomembranes by lipid peroxidation may be a major process mediated by activated oxygen radicals, leading to the induction of POD activity in maize roots. POD activity can be induced by a variety of other stress factors than metals (Vangronsveld and Clijsters, 1994). In our study, plants were cultivated in standardized climatic conditions, and wouding or pathogenic infection was avoided. The toxic threshold values calculated from POD activity vs. Cu concentration for roots and L3 were in the same order whereas the L4 ones was lower. This may indicate that young plant tissues are more sensitive to Cu than older ones. It could be related to an effect of Cu on metabolism in young cells and their lower possibilities to compartmentalize Cu. In addition, Cu might also interfere with the -SH groups involved in the catalytic action or structural integrity of enzymes, in particular those related to photosynthesis. Decrease in the photophosphorylation efficiency was reported in beans after application of excess Cu (Gora et al., 1985). Inhibition of carboxylase activity by 10% has been observed after in vitro incubation of Rubisco with 50 μM Cu (Van Assche and Clijsters, 1990a). The most interesting difference between maize roots and leaves was the presence of a new isoenzyme peroxidase in soluble proteins extracted from roots when Cu in HNS exceeded 0.5 μM. The critical value for the synthesis of this (iso)peroxidase was around 12.6 mg Cu kg DM^{-1} for Cu content in maize roots. This corresponds approximately to the toxic threshold value for POD and changes in chloroplast pigments in L4 although this (iso)peroxidase was not detected in this plant organ. A similar result was

obtained in beans (Van Assche and Clijsters, 1987): two isozyme bands appeared in the root extracts of bean seedlings treated with moderately toxic amounts of Cu (inducing 10% shoot growth inhibition) or cultivated on soils containing high amounts of Cu and Zn due to pig manure application, whereas in leaves they could only be detected when extremely toxic Cu concentrations (causing more than 50% shoot growth inhibition) were applied. The physiological meaning of isoenzyme induction is rather speculative. Appearance of new isoforms is generally considered as an important criterium for senescence (Vangronsveld and Clijsters, 1994).

In the roots the activity of the other investigated enzymes of the intermediary metabolism enhanced as a function of the tissue Cu content similar to the changes in POD activity. However, in the leaves, especially in L4, the activity of these enzymes decreased. The stimulation of ICDH and ME activity in roots (Figure 6) could be due to a demand for ATP production through oxidative phosphorylation (Van Assche and Clijsters, 1990a). Increase in GDH activity in maize roots may be related to the slight increase in root protein extracts, because this enzyme has a key role in amino acid metabolism (Van Assche and Clijsters, 1990a). Increase in ICDH and malate dehydrogenase (MDH) activities by Cu was also reported in bean roots by Weigel and Jäger (1980). Decrease in ICDH and G-6-PDH activity in L4 (Figure 5) might be explained by a lower protein synthesis rate but this cannot definitively be pointed out.

Data collected by Macnicol and Beckett (1985) suggest that if the tissue Cu concentration of any crop lies below 10 mg kg DW^{-1}, this element is not causing a reduction in yield. The critical values (i.e. tissue concentration at which yield is reduced by 10%) set up for the aerial parts of maize grown in soil widely varied in the literature from 5 to 21 mg Cu kg DW^{-1} (Macnicol and Beckett, 1985). The present study lead to even higher values (Figure 6). Toxic threshold value set up in L3 from changes in POD was near the upper value of this range. However, the threshold value accounting for leaf yield was even much more higher (Table 1, Figures 3b–3d).

Changes in mineral composition in relation to Cu content can be compared with data from literature for maize grown in nutrient solution. No changes in Ca and K contents as well as decrease in Fe content in roots was reported for 15 days-old maize plants cultivated in a Cu concentration range between 0.5 μM and 4.5 μM, with 32 mg Cu and 299 mg Cu per kg DW in roots (Ouzounidou et al., 1995). Phosphate content in both maize roots and shoots as well as iron content in shoots decreased at pH 4.7 when Cu raised from 0.16 to 3.1 μM Cu (Lexmond and van der Vorm, 1981). In our conditions, no significant change was observed in roots for Ca, K, P and Fe (Table 2), even though a slight decrease of root yield was observed for 10 μM Cu. Thus ion leakage appears unlikely. P content slightly decreased in L3 whereas Fe content numerically increased in L4. Trends in DM yield decrease for L3 and L4 in relation to Cu accumulation may explain the observed increase in Ca and Mg contents.

In conclusion, our results demonstrate that for maize plants cultivated on HNS with increasing Cu concentration, there is a transition from a likely healthy to a phytotoxic phase. This transition can not be detected using the current morphological parameters classically used by agronomists. The chloroplast pigments appear to be affected by Cu in the youngest leaf (i.e. L4). Consequently photosynthesis might be inhibited by Cu. However, in the same range for Cu concentration in plant tissue, very significant changes were noticed for other metabolic parameters. Total soluble protein content was not significantly affected in the 3rd-leaf and roots, but the increase in tissue Cu content led to large modification in enzyme activities, especially for POD. It appears that POD activity could be used as biomarker for sublethal Cu toxicity in maize because there is a dose-response relationship in all plant organs under investigation. This relationship needs to be confirmed with maize plants cultivated on Cu polluted soils such as acid coarse sandy soils from vineyard or orchard. The most interesting tool seems the study of the peroxidase isoenzyme pattern in soluble protein extracts of roots. The induction of a new (iso)peroxidase by Cu needs further investigation, especially to precise its specificity towards this metal. The relationships found between either POD or some NAD(P)H enzymes and Cu content in maize are also encouraging to investigate other enzymes involved in the detoxification of active oxygen radicals and aging processes such as catalase, SOD, and glutathione reductase.

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