

# Accumulation of low-molecular-weight organic acids in roots and leaf segments of *Zea mays* plants treated with cadmium and copper

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**Abstract** Maize seedlings grown in a nutrient solution were treated with Cd (50, 100  $\mu\text{M}$ ) or Cu (50, 100  $\mu\text{M}$ ). Roots and separated leaf sections (from the youngest—basal, through the middle—mature, to the oldest—apical) were analysed. Shoot and root fresh weight, and root net growth rates were reduced significantly after Cu application in comparison to Cd. Copper (50, 100  $\mu\text{M}$ ) and cadmium (100  $\mu\text{M}$ ) decreased the sum of chlorophyll and total carotenoid pools mostly in mature and old leaf sections. The concentration of Cu and Cd increased in the old and mature leaf sections. Analysis of organic acids showed that both metals differently influenced the low-molecular-weight organic acids (LMWOAs) content in maize leaf sections. In mature sections, the excess Cu increased the succinate and tartrate contents, whereas in the young ones mainly the tartrate level was elevated. Cadmium elevated citrate accumulation in mature and old sections, compared to the control plants. Malate, the main LMWOA in maize leaves, increased only after addition of 100  $\mu\text{M}$  of Cd (mature and old sections) or 50  $\mu\text{M}$  of Cu (old sections). Analysis of LMWOAs in roots showed that

the excess of Cd or Cu induced higher accumulation of tartrate and malate and, additionally, copper increased the citrate content.

**Keywords** Heavy metals · Maize · Organic acids · Malondialdehyde · Capillary electrophoresis

## Abbreviations

AA	Ascorbate
ANOVA	Analysis of variance
CTAB	Cetyltrimethylammonium bromide
FW	Fresh weight
GSH	Glutathione
HM	Heavy metal
LMWOA	Low molecular weight organic acid
LSD	Least significance difference
MDA	Malondialdehyde
NGR	Net growth rates
PC	Phytochelatin
PCA	Principal component analysis
PDC	2,6-Pyridinedicarboxylic acid
REL	Relative electrolyte leakage
ROS	Reactive oxygen species
TBA	2-Thiobarbituric acid
TCA	Trichloroacetic acid

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## Introduction

Although some of the heavy metals (HMs) such as iron, zinc, or copper are essential for normal growth, their presence at excess concentrations in soil becomes toxic to plants. These trace elements as well as non-essential HMs

such as lead or cadmium induce several mechanisms of plant adaptation to HMs stress. One effective way of HM stress reduction is the mechanism of extracellular HM detoxification by reducing the concentration of HMs entering the cell. On the other hand, when HMs enter the cytosol, intercellular mechanisms are involved in detoxification thereof. In such a situation, detoxification of HMs including an efflux of HMs from root cells due to H<sup>+</sup>-coupled antiport activity (Burzyński et al. 2005), metal transport, chelation, and sequestration into vacuole was observed (Rausser 1999; Sanità di Toppi and Gabbriellini 1999). Low-molecular-weight organic acids (LMWOAs) participate in both strategies of HMs detoxification: firstly, as plant exudates, organic acids increase extracellular precipitation of HMs by the chelation oxidation–reduction reaction in the rhizosphere; secondly, likewise phytochelatin (PCs) and amino acids, LMWOAs chelate and sequester HMs in the vacuole (Rausser 1999; Ma 2000; Ryan et al. 2001).

Previous papers indicate that LMWOAs such as malate, citrate, and oxalate can form a metal–organic acid complex in the vacuole (reviewed in Rausser 1999; Clemens 2001). This mode of detoxification was evidenced at the excess of Al (Ma 2000), Zn (Thurman and Rankin 1982; Harmens et al. 1994), and Ni (Lee et al. 1978; Yang et al. 1997) in nutrient medium.

Although the role of LMWOAs in detoxification of some HMs was well recognised, participation of LMWOAs in changing the toxicity of Cd and Cu excess in plants is still poorly understood. Chaffai et al. (2006) suggested that the role of LMWOAs in Cd and Cu detoxification is based on the internal complexation and compartmentation in the vacuole rather than on extracellular mechanism. According to these authors, increasing accumulation of citrate and malate in maize plants growing in excess of Cu or Cd suggests importance of these acids in internal HMs detoxification. Yang et al. (2005) indicated that citrate has strong affinity for Cd and Zn in leaves. In turn, citrate is one of the principal ligands for Cu, Ni, and Zn in the xylem sap moving from the roots upwards into the leaves. Additional difficulty in recognizing the role of LMWOAs in excess of Cu and Cd is the distinct stress response depending on the plant cell age. It is commonly known that plant response to Cd and Cu depends on plant age (Maksymiec and Baszyński 1996a, b; Skórzyńska-Polit and Baszyński 1997; Krupa and Moniak 1998; Dresler and Maksymiec 2013). Previous studies on the age-dependent response of monocotyledon leaf segments to Cd stress showed that Cd reduced photochemical processes more efficiently in older than younger leaf sections (Drażkiewicz et al. 2003). Additionally, in Cd-exposed plants, various phytochelatin concentrations were discovered in relation to leaf segments and Cd concentration (Drażkiewicz et al.

2003; Drażkiewicz and Baszyński 2005). Dresler and Maksymiec (2013) observed various ascorbate (AA) and glutathione (GSH) concentrations related to the Cd or Cu plant treatment and section of maize leaf. According to these authors, Cu excess increased GSH and glutathione disulphide in all the leaf sections and AA in old and mature sections, while GSH increased in old leaf sections and decreased in young leaf sections in Cd-exposed plants. Furthermore, the concentration of AA in the Cd-treated plants was similar to the control.

In the present study, we investigate in a short time experiment the effects of acute stress caused by high Cd and Cu concentrations on maize plants and the response and LMWOA accumulation in roots and in different-aged segments of leaves. Moreover, the Cd and Cu effect on the growth, photosynthetic pigments, malondialdehyde (MDA) content, relative electrolyte leakage (REL) and the content of Cu and Cd in plant tissues are presented.

## Materials and methods

### Plant material

Seeds of maize (*Zea mays* L. cv. Reduta) were germinated for 2 days on wet filter paper in a thermostat-controlled chamber. Similar looking seedlings (about 1 cm long germ and 5–8 cm long roots) were transferred into Hoagland full strength nutrient solution. After adaptation for 2 days (4 days from germination), the nutrient solution was changed and the plants were treated with Cd (50, 100 μM) or Cu (50, 100 μM). Cadmium and Cu were added as Cd(NO<sub>3</sub>)<sub>2</sub> and CuSO<sub>4</sub> (Sigma), respectively. The control plants remained without Cu or Cd addition. The plants were grown for another 8 days at a day/night cycle of 16/8 h and 24/17 °C, respectively, relative humidity 60–70 %, and photosynthetic photon flux density of 150 μmol m<sup>-2</sup> s<sup>-1</sup>. The nutrient solution for control plants, as well as that containing Cd or excess Cu, was changed after 4 days. After 8 days from metal treatment (12 days from germination), the plants were divided into shoots and roots, and the fresh mass of shoot and roots was weighed. The third leaf was sampled and cut into three sections of equal length from the basal (young) through the middle (mature) to the apical part (old) (Fig. 1). Furthermore, roots were collected after thorough washing with distilled water. The primary root net growth rates (NGR) during the 10 days of the Cd treatment were calculated as follows: (final length—initial length)/initial length).

### Organic acid estimation

Fresh plant tissue samples (aliquots of 50–200 mg part of leaf or root) were extracted by homogenisation in distilled water (2 ml). The homogenate was incubated for 30 min at



**Fig. 1** Sections of the third leaf of *Zea mays* growing for 12 days

50 °C. After cooling, the mixture was centrifuged at  $10,000\times g$  for 5 min. Finally, before sample injection into the CE system, the supernatant was filtered through 0.20  $\mu\text{m}$  nylon filters. Measurements were performed on an Agilent 7,000 capillary electrophoresis system (Agilent Technologies) equipped with a diode array spectrophotometric detector (190–600 nm). The capillary electrophoresis separation was performed according to the method described by Soga and Ross (1997). Briefly, a fused-silica capillary with 75  $\mu\text{m}$  id with effective lengths of 72 cm (80.5 cm total length) (Agilent Technologies) was used. The applied voltage was set at  $-25$  kV (negative polarity mode). The samples were injected by pressure (50 mbar) for 4.0 s. Prior to first use, new capillaries were rinsed with run buffer under pressure (approximately with 1 bar) for 15 min. Before each injection, the capillary was preconditioned for 4 min by flushing with the run electrolyte. Background electrolytes contained 5 mM 2,6-pyridinedicarboxylic acid (PDC, Sigma) and 0.5 mM cetyltrimethylammonium bromide (CTAB, Sigma), which were used to reverse the direction of electroosmotic flow. The pH was adjusted to 5.6 with 1 M NaOH. The data obtained were analysed using ChemStation data analysis software (3D-CE ChemStation, Agilent Technologies).

#### Lipid peroxidation assay

For MDA determination, 0.5 g of each plant leaf section or roots was homogenised in 0.1 % trichloroacetic acid (TCA) and centrifuged at  $10,000\times g$  for 10 min. 0.5 % 2-thiobarbituric acid (TBA) in 20 % TCA was added to the supernatant. The mixture was heated for 30 min at 95 °C, quickly cooled, and centrifuged at  $10,000\times g$  for 10 min. The absorbance of the supernatant was read at 532 and 600 nm (Tewari et al. 2002). The level of lipid peroxidation is defined as the MDA content.

#### Analysis of relative electrolyte leakage

Leaf disks of 6-mm diameter each (0.2 g) from fresh plants were washed for 3 min with ultrapure water (40 ml) (MilliQ, Millipore, USA). Next, the leaf disks were dried with filter paper, placed in test tubes containing ultrapure water (10 ml) and shaken for 3 h at 25 °C to measure

initial conductivity (L1) (MPC227 meter, Mettler Toledo, Switzerland). Afterwards, the leaf disks were heated for 20 min at 95 °C to measure final conductivity (L2). REL was calculated as  $\text{REL} (\%) = (L1/L2) \times 100$ .

#### Chlorophyll and carotenoid content

Chlorophyll ( $a + b$ ) and carotenoids were extracted in 80 % acetone and calculated according to Wellburn (1994).

#### Copper and cadmium content

For copper and cadmium determination, the root (after rinsing, incubated in ice-cooled 10 mM  $\text{CaCl}_2$  solution for 30 min) and sections of leaf samples were dried at 105 °C to a constant dry weight. Afterwards, the dry material was wet digested in mixed acids [ $\text{HNO}_3 + \text{HClO}_4$  (4:1, v/v), Sigma]. The cadmium and copper concentrations were measured by atomic absorption spectrophotometry.

#### Statistics analysis

Principal component analysis (PCA) of the LMWOA content and growth parameters of control and Cd- and Cu-treated plants was carried out. Using analysis of variance (two-way ANOVA), the effects of excess Cu or Cd on growth parameters, LMWOAs, MDA, REL, and photosynthetic pigments content in the leaf sections were analysed. Differences between the levels were determined with Fisher's least significance difference test at the 0.05 probability level. The data were analysed using Statistica ver. 6 (StatSoft, Inc. 2004).

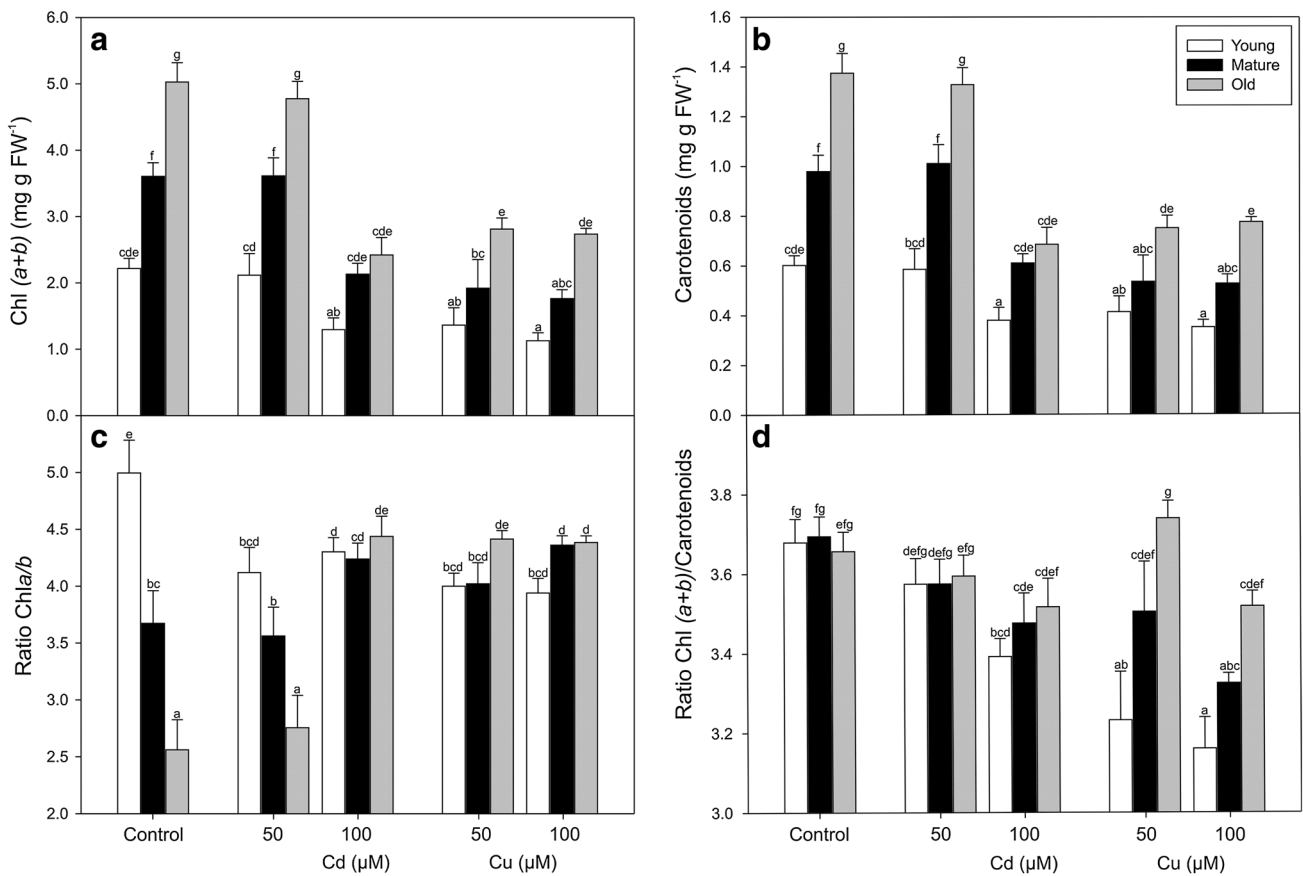
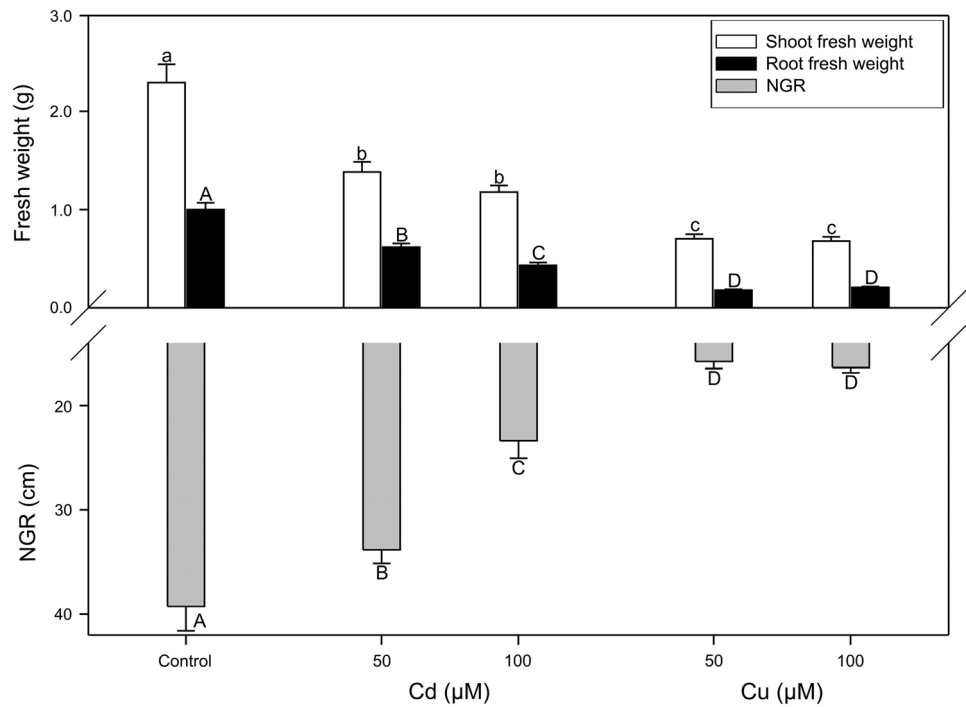
## Results

#### Growth parameters and content of photosynthetic pigments

In the present investigation, both Cd and Cu significantly reduced the fresh biomass. The 50 or 100  $\mu\text{M}$  cadmium concentration reduced the aboveground part weight approximately by 40 and 50 % and the root weight by 34 and 57 %, respectively. Excess Cu reduced the weight of shoots by  $\sim 70$  % and roots by 80 % (both Cu concentration). After Cd treatment, NGR decreased by 15 and 40 % (50 and 100  $\mu\text{M}$ , respectively) and Cu by 60 % (Fig. 2).

The content of photosynthetic pigments (expressed as  $\text{mg g}^{-1}$  FW of leaf section) depended on the age of each section and the concentration of Cd or Cu in the nutrient solution (Fig. 3a, b). The control and heavy metal-treated maize plants accumulated the greatest amount of pigments

**Fig. 2** The effect of Cd and Cu on growth parameters of maize seedlings. Mean ( $\pm$ SE) with the different letters within the same growth parameter are significantly different according to Fisher's test ( $p < 0.05$ ) ( $n = 12$ )



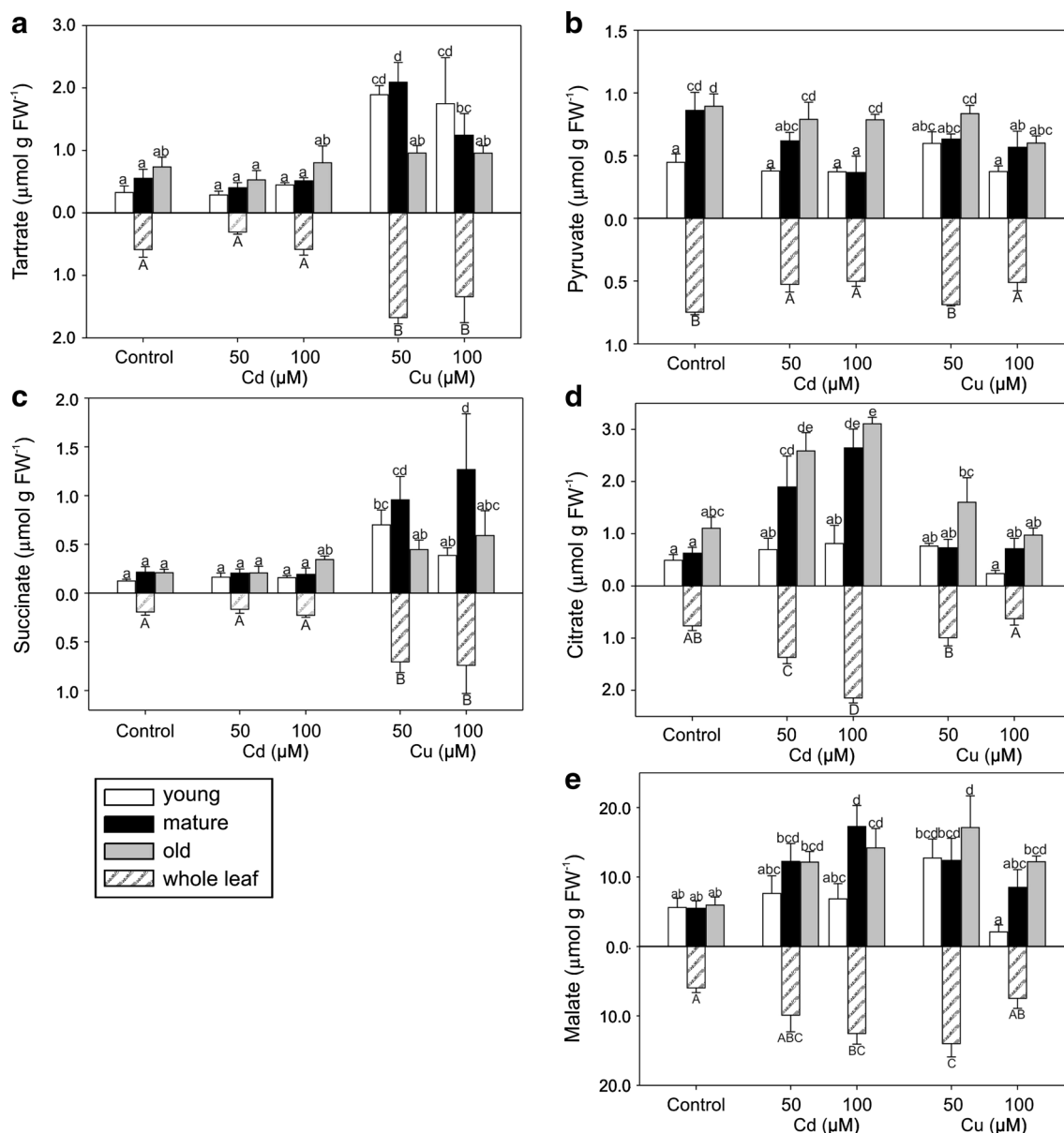
**Fig. 3** Effect of Cd and Cu on the content of photosynthetic pigments in leaf sections differing in age of *Zea mays* seedlings. Mean ( $\pm$ SE) with the different letters within the same parameter are significantly different according to Fisher's test ( $p < 0.05$ ) ( $n = 4$ )

in the oldest leaf sections. However, after 50  $\mu\text{M}$  of Cu and 100  $\mu\text{M}$  of Cu or Cd treatments, the sum of chlorophyll and total carotenoid pools decreased mostly in mature and old leaf sections. The chlorophyll  $a/b$  ratio in maize leaf sections ranged between 2.6 and 5.0 (Fig. 3c). However, Cd and Cu stress significantly decreased the  $\text{Chl}a/b$  ratio in young leaf segments and increased the value of the parameter in the 50 or 100  $\mu\text{M}$  Cu or 100  $\mu\text{M}$  Cd variant in the older sections. Figure 3d illustrates the changes in the ratio of the sum of chlorophyll ( $a + b$ ) to total carotenoids that occurred in leaf segments in the Cd or Cu treatments. It was observed that the ratio of  $\text{Chl}(a + b)/\text{Car}$  increased with increasing age of leaf segments exposed to Cu, whilst

in the control and cadmium treatment the ratios of  $\text{Chl}(a + b)/\text{Car}$  were similar in all the leaf sections.

#### Organic acid accumulation

Five types of LMWOAs were identified in the leaf sections and four in the root extracts (the concentration of pyruvate in roots of maize was under the limit of detection) of *Zea mays* plants (Fig. 4; Table 1). Tartrate and succinate content in the shoots of the control and treated maize plants showed a similar tendency to accumulation (Fig. 4a, c). The concentration of these acids increased in the whole leaf of maize exposed to Cu in comparison with the control and



**Fig. 4** Effect of Cu and Cd on LMWOAs concentration in *Zea mays* leaf segments. Mean ( $\pm$ SE) with the different letters within the same parameter are significantly different according to Fisher's test ( $p < 0.05$ ) ( $n = 4$ )

**Table 1** Effect of Cu and Cd on organic acids ( $\mu\text{mol g f wt}^{-1}$ ) ( $n = 4$ ) and MDA ( $n = 12$ ) ( $\text{nmol g f wt}^{-1}$ ) content in roots of *Zea mays*

	Tartrate	Malate	Citrate	Succinate	MDA
Control	0.216 $\pm$ 0.083b	0.392 $\pm$ 0.088a	0.069 $\pm$ 0.029a	0.049 $\pm$ 0.032a	2.23 $\pm$ 0.055a
Cd					
50 $\mu\text{M}$	1.075 $\pm$ 0.177a	1.606 $\pm$ 0.360bc	0.370 $\pm$ 0.071a	0.169 $\pm$ 0.014b	3.15 $\pm$ 0.408ab
100 $\mu\text{M}$	0.786 $\pm$ 0.114a	0.851 $\pm$ 0.080ab	0.189 $\pm$ 0.043a	0.095 $\pm$ 0.034ab	3.27 $\pm$ 0.448ab
Cu					
50 $\mu\text{M}$	1.098 $\pm$ 0.122a	3.336 $\pm$ 0.458c	0.769 $\pm$ 0.077b	0.141 $\pm$ 0.029ab	8.90 $\pm$ 2.554c
100 $\mu\text{M}$	0.640 $\pm$ 0.239ab	2.095 $\pm$ 0.563d	0.837 $\pm$ 0.334b	0.138 $\pm$ 0.059ab	6.23 $\pm$ 0.893bc

The values are mean  $\pm$  SE

Different letters denote significant differences between the treatments at  $p < 0.05$

Cd-treated plants. Cu excess increased succinate levels mainly in the mature sections of the leaves, whereas in the lowest Cu concentration increased the tartrate content about sixfold in young and fourfold in mature leaf segments compared to the control. In the oldest leaf sections, the concentration of that acid was not significantly different between the treatments. By contrast, the citrate level significantly increased in mature and old leaf sections in the Cd-exposed plants (304, 427 %—for mature, and 235, 282 %—for old, compared to the control at 50 or 100  $\mu\text{M}$ , respectively) (Fig. 4d). Along with leaf ageing, the pyruvate concentration showed a tendency to increase; however, this effect was not significant in the tested plants (Fig. 4b). The main organic acid in the leaves of *Zea mays* plants was malate (Fig. 4e). However, its content exhibited several differences related to leaf cell age and the Cu or Cd treatment and accounted for approximately from 40 to 80 % of the total acid content. Although 100  $\mu\text{M}$  Cu or 50  $\mu\text{M}$  Cd did not have a significant effect on the malate concentration in the leaf segments, a tendency for malate to increase in mature and old sections was noted. A similar significant effect was observed in the case of 100  $\mu\text{M}$  Cd or 50  $\mu\text{M}$  Cu. Cadmium and Cu excess increased the malate concentration approximately threefold both in the mature leaf segments growing at 100  $\mu\text{M}$  Cd and in the old leaf section at 50  $\mu\text{M}$  Cu compared to the control.

As seen from Table 1, the content of organic acids in roots after the action of Cd or Cu had a tendency to increase. Fifty or 100  $\mu\text{M}$  Cd and 50  $\mu\text{M}$  Cu significantly elevated the tartrate concentration. In the case of malate, a similar increase was detected after application of Cu (both concentrations) or 50  $\mu\text{M}$  Cd. Although the citrate concentration in shoots did not increase after Cu action, in roots it reached 1,100 and 1,200 % of the control at 50 or 100  $\mu\text{M}$  Cu, respectively. In contrast to the shoot results, exposure to Cd had no significant effect on the citrate content in roots. The succinate content in roots showed a slight increase in response to Cd or Cu; however, a significant increase was observed only in 50  $\mu\text{M}$  Cd.

The PCA of the organic acid concentrations and growth parameters in the plants studied (Fig. 5) showed clear separation between the control and plants treated with Cd and Cu. The first two PCs explained 47.7 % of the total variance. The first component (PC1—30.2 % of the variation) is largely negatively determined by shoot and root fresh weight. Additionally, the PC1 has strong positive loadings on the malate and citrate concentration in roots and tartrate and succinate in all the three leaf sections examined. The PC1 separated the Cu effect from the control and Cd-treated plants. On the other hand, the second component (PC2), which accounted for 17.5 % of the variation, was mainly explained by the content of malate and citrate in leaves and tartrate in roots.

#### Lipid peroxidation and relative electrolyte leakage level

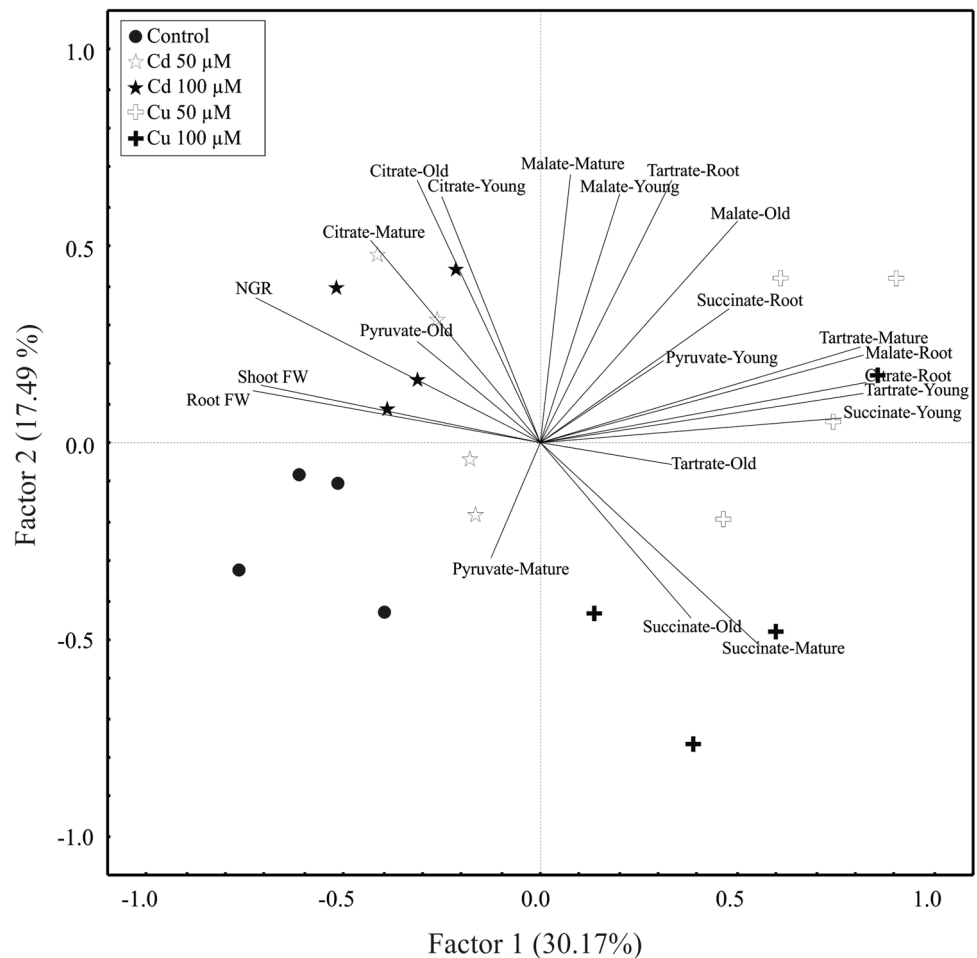
Our results showed that the level of lipid peroxidation of cell membranes increased with the age of leaf sections (Table 2). In the presence of 50  $\mu\text{M}$  Cd, MDA in the leaf sections increased significantly about 4-, 3- and 2.5-fold in the young, mature, and old leaf sections, respectively. However, when the plants were exposed to 100  $\mu\text{M}$  Cd or Cu (both 50 and 100  $\mu\text{M}$  concentrations), the levels of this parameter were similar to the control. The MDA levels in the roots of the Cd-treated plants were similar to the control, while 50 or 100  $\mu\text{M}$  Cu increased the MDA level approximately 4- and 3-fold, respectively.

Table 3 shows the REL value in the leaf sections. The results obtained indicate that electrolyte leakage was increased in the young leaf sections in all the treatments as well as in the control.

#### Copper and cadmium content

The concentrations of Cu and Cd in leaf segments and roots are shown in Fig. 6. Both cations content showed an upward trend in the treated plants with the age of

**Fig. 5** Scatter plot of the PCA of LMWOAs concentrations in selected leaves sections and roots, and growth parameters variations in the *Zea mays* seedlings grown in control or exposed to Cu and Cd. Length of lines of the scatter plot overlay shows correlation between original data and PC axes



**Table 2** MDA (nmol g f wt<sup>-1</sup>) content in leaf segments of *Zea mays* seedlings treated with Cd and Cu ( $n = 12$ )

Leaf segments	Control	Cd		Cu	
		50 $\mu$ M	100 $\mu$ M	50 $\mu$ M	100 $\mu$ M
Young	27.31 $\pm$ 6.14ab	107.16 $\pm$ 4.18e	31.89 $\pm$ 6.06ab	16.16 $\pm$ 2.93a	37.88 $\pm$ 12.12ab
Mature	51.60 $\pm$ 9.66bcd	159.22 $\pm$ 1.98f	53.31 $\pm$ 11.58bcd	37.62 $\pm$ 2.63ab	42.86 $\pm$ 14.29abc
Old	67.93 $\pm$ 7.86 cd	162.24 $\pm$ 11.64f	76.36 $\pm$ 12.31d	51.56 $\pm$ 19.71bcd	53.42 $\pm$ 9.86bcd

The values are mean  $\pm$  SE

Different letters denote significant differences between the treatments at  $p < 0.05$

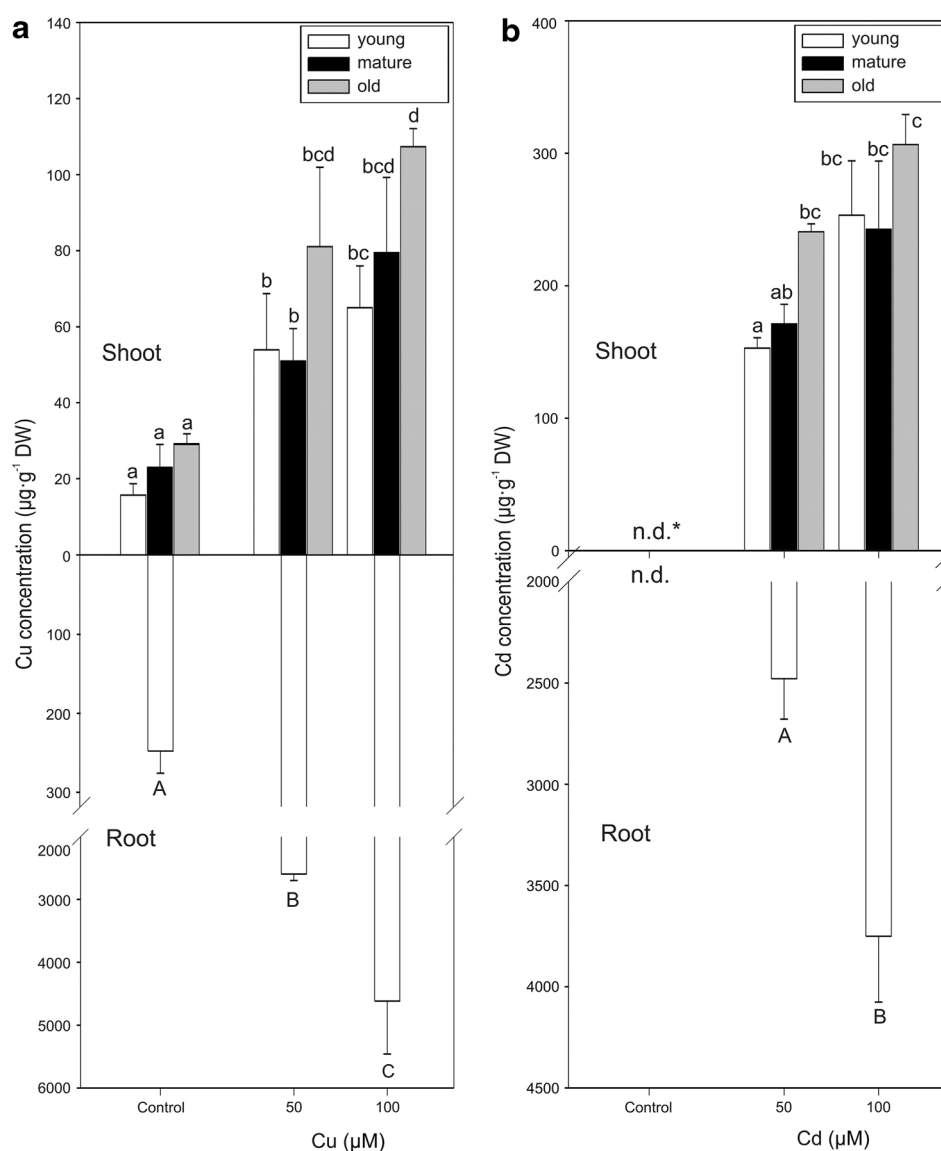
**Table 3** REL value in leaf segments of *Zea mays* seedlings treated with Cd and Cu ( $n = 12$ )

Leaf segments	Control	Cd		Cu	
		50 $\mu$ M	100 $\mu$ M	50 $\mu$ M	100 $\mu$ M
Young	17.22 $\pm$ 1.27c	12.10 $\pm$ 1.12a	12.86 $\pm$ 0.99ab	15.72 $\pm$ 1.61bc	13.03 $\pm$ 0.66ab
Mature	12.53 $\pm$ 0.88ab	10.82 $\pm$ 0.92a	11.21 $\pm$ 1.04a	13.05 $\pm$ 1.23ab	12.59 $\pm$ 0.96ab
Old	10.51 $\pm$ 1.15a	11.18 $\pm$ 1.84a	9.51 $\pm$ 0.84a	12.09 $\pm$ 0.98ab	12.93 $\pm$ 3.15ab

The values are mean  $\pm$  SE

Different letters denote significant differences between the treatments at  $p < 0.05$

**Fig. 6** The mean of the Cu (a) and Cd (b) content in *Zea mays* leaf segments and roots grown under copper or cadmium treatment for 8 days. Mean ( $\pm$ SE) with the different letters are significantly different according to Fisher's test ( $p < 0.05$ ) ( $n = 3$ )



segments. The level of both metals in the leaf sections did not change significantly depending on the metal concentration in the nutrient medium. But, a significant increase was observed in the oldest sections after 100  $\mu\text{M}$  Cu or 50  $\mu\text{M}$  Cd treatments. The content of Cu and Cd in roots increased significantly with the increase in the concentration of the metals in the growth medium.

## Discussion

LMWOAs play an important role in several biochemical pathways, including respiration and energy generation, amino-acid biosynthesis, photosynthesis, cation transport, and reduction of HM stress (Cataldo et al. 1988; López-Bucio et al. 2000; Schulze et al. 2002). The hypothesis about the participation of LMWOAs in detoxification of HMs was proposed by

Rauser (1999) based on previous study on accumulation of LMWOAs in plant tissues of various HM-tolerant plant clones. There is substantial evidence indicating a close relationship between the LMWOA content and tolerance to HMs (Thurman and Rankin 1982; Rauser 1999; Ma 2000; Boominathan and Doran 2003). However, the role of LMWOAs in detoxification of HMs is still being discussed (Hall 2002; Wójcik 2009).

The accumulation of LMWOAs in Cu- and Cd-stressed plant tissues has been previously described by a few authors (Krotz et al. 1989; Chaffai et al. 2006; Wójcik et al. 2006); however, to our knowledge, the Cu and Cd influence on the concentration of LMWOAs in different leaf developmental stages has not been investigated.

In the present study, the strong Cu and Cd effect on LMWOA accumulation in leaves and roots was observed. The results obtained indicated that excess Cu increased tartrate, succinate, and malate levels, while Cd had a



positive effect on citrate and malate in leaves. Similar results have been previously reported by Chaffai et al. (2006). According to these authors, in response to Cd and Cu stress, maize roots accumulate more citrate, while leaves accumulate malate at Cu excess and citrate at Cd treatment.

Moreover, our results indicate that Cd (both 50 and 100  $\mu\text{M}$ ) had a positive influence on the citrate concentration only in the mature and old leaf sections. The result implies that citrate and malate (at high Cd concentrations) probably play a role in Cd transport and sequestration in old leaves while malate participates in Cu (50  $\mu\text{M}$  Cu concentration) detoxification in old leaf sections. This conclusion reinforces the hypothesis that citric acid is one of the Cd ligands and participates in Cd transport in xylem sap to the oldest part of plants (Cataldo et al. 1988, reviewed Clemens 2001). Previously (Krupa and Moniak 1998; Drązkiewicz et al. 2003), the lowest level of Cd was noted in young leaf sections of monocotyledonous plants, while mature and old sections retained the HM concentration at a high level. The afore-mentioned studies are in accordance with our results, however, only in 100  $\mu\text{M}$  Cu and 50  $\mu\text{M}$  Cd treatments, where significantly different Cu or Cd concentrations between young and old sections were observed. The changes in Cu and Cd concentrations are similar to the values reported by Drązkiewicz et al. (2003) and Žaltauskaitė and Šliumpaitė (2013). Higher Cu and Cd concentrations in roots than in shoots are commonly known in the literature (Tukendorf and Baszyński 1985; Chaffai et al. 2006). Drązkiewicz et al. (2003) indicated that lower Cd shoot concentrations could result from compartmentation of the metal with sulphur ligands in roots cells and restricted their transport into the above-ground parts of seedlings. Additionally, the higher concentration of LMWOAs obtained in metal-treated roots indicated possibilities of compartmentation of HMs by LMWOAs in roots.

Although there is some evidence that citrate participates in Cd complexation, comparison of the molar concentration of both acids (malate and citrate) in the leaves indicated that the content of the former acid was approximately tenfold greater than that of the latter. This observation shows that among LMWOAs malate plays a crucial role in detoxification of Cd and Cu. However, the malate level increased significantly only after 100  $\mu\text{M}$  of Cd (mature and old sections) or 50  $\mu\text{M}$  of Cu (old sections), which may indicate that the lower concentration of Cd was not sufficiently toxic to induce malate synthesis while the strong toxic effect of Cu (100  $\mu\text{M}$ ) disturbed accumulation of malate. Wójcik et al. (2006) reported that malate was a predominant LMWOA in shoots and roots of *T. caerulea*. Moreover, Cd and Zn stimulated malate accumulation in shoots while citrate was found only in Zn-treated shoots.

Since the concentration of pyruvate in Cu- or Cd-exposed maize leaves was similar to that in the control, determination of its role in HM tolerance was difficult. Interestingly, only Cu increased accumulation of succinate and tartrate in younger parts of leaves. This evidence could point out that those acids participate in Cu detoxification. The results of the study by Doncheva et al. (2006) demonstrated that exogenous succinate decreased the toxic effect of Cu on maize growth, photosynthesis pigment content, and activities of antioxidant enzymes. Furthermore, the authors confirmed the high affinity of succinate to Cu. However, the higher concentration of these LMWOAs (succinate and tartrate) in the younger leaf sections under Cu stress indicates a different mechanism of Cu detoxification by malate and/or Cd detoxification by citrate. The probable clue could be the limitation of translocation to the older leaf section. Nevertheless, one cannot exclude the possibility that the succinate and tartrate rise in Cu-treated leaves was the result of a negative effect on the physiological process. We have shown that the growth of shoot and roots was more limited by Cu than by Cd. Additionally, photosynthetic pigments that are considered as markers of plant physiological state were significantly decreased in Cu (both Cu concentrations) compared to the control and 50  $\mu\text{M}$  Cd-treated plants. Thus, the high toxicity effect of Cu could disturb the physiological pathways in which LMWOAs participate; therefore, the concentration of some of them was increased in the young leaf sections.

The age-dependent differences observed in the concentration of LMWOAs in Cu- and Cd-treated leaves were accompanied by definite changes in the Chl $a/b$  and Chl $(a + b)/\text{Carot}$  ratios, compared to the control. The increase in the Chl $a/b$  ratio in young and mature Cu-treated leaves (50 or 100  $\mu\text{M}$  Cu) or Cd-treated leaves (100  $\mu\text{M}$ ) points out that those metals disturbed the antennae pigments, which are related to greater sensitivity of Chl $a$  than Chl $b$  to HMs (Drązkiewicz and Baszyński 2005). It is known that HMs interfere with iron metabolism causing both iron deficiency and a decrease in chlorophyll synthesis, which was observed after 100  $\mu\text{M}$  Cd, 50 or 100  $\mu\text{M}$  Cu supplementation. Additionally, the level of carotenoids decreased in the treatments mentioned above and they were not able to protect plants against 50 or 100  $\mu\text{M}$  Cu or 100  $\mu\text{M}$  Cd.

Our results indicated that the Cu in excess exerted a more toxic effect than Cd on plant growth, which is in accordance with previous studies (Chaffai et al. 2006; Žaltauskaitė and Šliumpaitė 2013); in contrast, Metwali et al. (2013) and Gupta and Abdullah (2011) did not observe such toxic effects of Cu. Surprisingly, REL was not increased in the leaf segments in Cd or Cu excess,

while MDA increased only in the roots of the Cu-treated plants and in the leaves of 50  $\mu\text{M}$  Cd-treated maize. The increased MDA level could be connected with both increased  $\text{H}_2\text{O}_2$  concentration and Fe efficiency. In some experiments, the induction of MDA, an oxidative marker, is more pronounced in roots than in leaves after metal stress, for example in Cu-stressed rice seedlings (Mostofa and Fujita 2013). In the case of Cd stress, the increase in the MDA content was similar in leaves and roots (Cao et al. 2013). In our study, the level of stress monitored by the MDA level was more pronounced in senescent *Zea mays* leaves and more visible in the leaves after Cd treatment and in the roots after Cu application.

Furthermore, the MDA concentration was higher at the lower Cd dose. Hydroperoxides formed from lipids may have been preferentially converted at the higher Cd concentration into more stable compounds, including jasmonic acid, as was supposed by Rucinska and Gwózdź (2005) and supported by the findings of Maksymiec et al. (2005), which indicated rapid accumulation of jasmonic acid after heavy metal stress. The lack of significant changes in the membrane permeability additionally confirms this conclusion. After application of 50  $\mu\text{M}$  Cd, an especially high MDA level was detected, and no changes in membrane permeability were observed. Our results indicate that lipid peroxidation cannot destabilise cell membrane and are in accordance with results presented by Li et al. (2012), but those results depended on different factors, e.g. time of treatments and plant age.

As mentioned above, the different effect of both metals on accumulation of LMWOAs as well as photosynthetic pigments, MDA, and growth parameters is dependent on the different toxicity effects of Cu and Cd. The excess of Cu induces oxidative damage by producing toxic free radicals; furthermore, Cu inhibits some of the enzymes implied in photosynthesis, whereas Cd has an ability to produce oxidative stress, but in contrast to Cu, it does not participate directly in the production of reactive oxygen species via the Fenton reaction.

In conclusion, we showed that (1) both metals influenced differently the accumulation of LMWOAs in roots and in individual sections of maize leaf, (2) increasing accumulation of malate under metal stress in mature and old leaf sections might indicate that it plays a crucial role in Cd (higher concentration) and Cu (lower concentration) detoxification, (3) the increasing citrate concentration in old and mature leaf sections after Cd exposure implies specific participation of the acid in Cd detoxification, (4) only Cu-exposed plants had an increased level of succinate and tartrate in the young leaf sections, (5) the Cu excess exerted a more toxic effect than Cd on plant growth and the content of photosynthetic pigments in maize seedlings.

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