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ORIGINAL PAPER

Induction of phenolic compounds in two dark-grown lentil cultivars with different tolerance to copper ions

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Abstract The influence of a high copper sulphate concentration on growth, Cu accumulation, lipid peroxidation as well as on the contents of total phenolic compounds (PhC) and UV-absorbing compounds (UVAC) in roots of lentil (*Lens culinars* Medic.) cvs. Krak and Tina was investigated. The plants were subjected to 0.5 mM Cu^{2+} for 3 and 5 days in darkness. Growth inhibition and increased lipid peroxidation in the roots of both cultivars, especially in cv. Tina which accumulated more Cu, were observed. Cu^{2+} treatment caused greater PhC and UVAC accumulation in cv. Krak; however, constitutive levels of these compounds were higher in cv. Tina. The maximum absorption peak of UVAC was determined at 270 nm. HPLC analyses of these compounds revealed the presence of two main derivatives of the soluble (aglycone and ester-bound) fraction of the hydroxycinnamic acids, ferulic (FA) and *p*-coumaric (*p*-CA) acids and the flavonol, kaempferol (Kam). Greater changes in the content of phenolic acids than of Kam may suggest that the former play a more important role in protecting lentil roots against high Cu^{2+} concentration. Thus, while the lower PhC levels at a higher Cu content in the roots of cv. Tina were probably due to stress, their higher levels in cv. Krak could have been a response to ROS signaling. However, though the high concentration of Cu^{2+} stimulated PhC in cv. Krak, it was not sufficient to counteract the amount of ROS generated by metal presence. These observations may suggest that ROS can serve as a common signal for acclimation to Cu^{2+} stress and cause PhC accumulation in dark-grown roots. The role of PhC in lentil tolerance to Cu^{2+} stress is discussed.

Keywords Copper ions · Root growth parameters · Hydroxycinnamic acids · Kaempferol · *Lens culinaris* Medic. · Lipid peroxidation · Total phenolic compounds · UV-absorbing substances

Abbreviations

FW	Fresh weight
DW	Dry weight
Kam	Kaempferol
p-CA	*p*-Coumaric acid
FA	Ferulic acid
PhC	Phenolic compounds
ROS	Reactive oxygen species
TBARS	Thiobarbituric acid reactive substances
UVAC	UV-absorbing compounds

Introduction

Copper (Cu) in small doses is an important microelement in plants, but at higher concentrations causes different physiological and biochemical disorders. The phytotoxic effects of excessive Cu^{2+} have been the subject of many studies (Maksymiec 1997, 2007). This redox-active metal at higher concentrations catalyzes the formation of harmful

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reactive oxygen species (ROS), such as superoxide, hydroxy peroxide and hydroxyl radicals, which can induce oxidative damage in important macromolecules such as DNA, proteins and lipids (Gaetke and Chow 2003). On the other hand, ROS can serve as a signal for acclimation to e.g. heavy metal stress (Babu et al. 2003; Maksymiec 2007). Toxic effect of Cu^{2+} resulting from the oxidative state may be allayed by several antioxidative systems including phenolic compounds (PhC), proline, tocopherols and polyamines (Mittler 2002; Grace 2005; Górecka et al. 2007).

It has been observed that PhC in higher plants can act as antioxidants and effectively prevent oxidative stress caused by environmental conditions such as low temperature (Janas et al. 2002), pathogen infections (Treutter 2006), UV radiation (Bieza and Lois 2001) as well as heavy metals, e.g. Cu^{2+} (Caldwell 2002; Sgherri et al. 2002; Jung et al. 2003; Michalak 2006; Górecka et al. 2007). It is possible that PhC acting as reductants may scavenge ROS or chelate heavy metals, thus decreasing metal toxicity in cells (Gordon and Roedig-Penmam 1998; Sgherri et al. 2002). In particular, their carboxyl or hydroxyl groups can strongly bind Cu^{2+} and Fe^{2+} (Fernandez et al. 2002). It should be emphasized that PhC (i.e. phenylpropanoids such as flavonoids and derivatives of hydroxycinnamic acids), in contrast to their antioxidant activity, can also act as prooxidants (Sakihama et al. 2002). The response of PhC to Cu^{2+} can vary among plant species and in different tissues as well as at varying metal concentrations (Caldwell 2002; Gordon and Roedig-Penmam 1998; Ali et al. 2006). Various plant species react differently to excess Cu^{2+}, but differences in plant responses to this metal ions seem to depend not only on its concentration but also on the capability of plants to increase the antioxidative protection against negative consequences of heavy metal stress.

PhC also play an important role in the control of many biological activities in plants, acting as, e.g. enzyme inhibitors, light-absorbing pigments, light screens, visual attractants for pollinators, regulators of plant growth, chemical signals in nodulation gene induction, as well as phytoalexins (Grace 2005). There is an emerging view that flavonoids may exert modulatory actions in cells through the influence on the protein kinase signalling pathways (Williams et al. 2004).

Lentil (*Lens culinaris* Medic.), which belongs to legumes, is an important plant in the human diet all over the world, being one of the best and cheapest sources of vegetable protein and a good source of minerals. Legume roots are a large source of PhC, especially hydroxycinnamic acids and flavonoids, which play a significant role in nitrogen-fixing symbiosis. They act as molecule signals, which induce the transcription of bacterial genes, where protein products are required for infection process (Rengel 2002). It was observed that some flavonoids that induce *nod* gene expression in rhizobia can also stimulate spore germination and hyphal growth of the arbuscular mycorrhizal fungi, which can alleviate metal toxicity, e.g. Cu^{2+} in the host (Rengel 2002).

Heavy metal contamination of agricultural soils caused by the application of pesticides and fertilizers is a serious environmental problem that can reduce the productivity of plants and inhibit the nodulation of leguminous species. It was observed that the nodulation process was more sensitive to increasing Cu^{2+} concentrations than both shoot and root growth (Kopittke et al. 2007).

Photosynthetically active tissues respond to UV light causing the same effect as Cu^{2+} induction of a similar type of PhC. Both types of environmental stresses cause an oxidative stress and induce ROS formation. Thus, ROS can serve as a signal for the enhancement of PhC synthesis and for acclimation to stress caused by both Cu^{2+} and UV radiation (Babu et al. 2003). It seems that both stresses can activate the same processes, although different receptors and signaling pathways are involved (Poschenrieder et al. 2006). Therefore, we supposed that Cu^{2+}-stressed dark-grown roots of lentil should accumulate PhC induced by ROS and their levels should depend on plant tolerance to metal.

Although reports about Cu^{2+} toxicity to plants do exist, there is no available information on the effects of Cu^{2+} on PhC accumulation in lentil (*Lens culinaris* Medic.) roots. Therefore, the objective of the present study was to determine the effects of Cu accumulation on lipid peroxidation in roots and correlative changes in PhC content in two lentil cultivars cultivated in darkness.

Materials and methods

Plant material and growth conditions

Lentil (*Lens culinaris* Medic.) cv. Krak seeds were obtained from Prof. H. Piróg (Department of Basic Agriculture, Agricultural University of Cracow, Poland) and cv. Tina seeds were obtained from the 'Spójnia' Plant Breeding and Seed Production Nochowo, Poland. The seeds were surface sterilized in a fungicide, Thiuram (Organica-Sarzyna, Poland) and were germinated at 25°C for 3 and 5 days in plastic boxes with a layer of cotton wool wetted with distilled water (control) or $CuSO_4$ water solution (0.5 mM). After 3 and 5 days of germination, the length of seedling roots was measured. The roots were weighed to record fresh weight (FW) and then dried in an oven at 60°C until it reached a constant dry mass (DW). All experiments were carried out in darkness.

Evaluation of lipid peroxidation

The level of lipid peroxidation products in the samples was evaluated as thiobarbituric acid reactive substances (TBARS), mainly malondialdehyde (MDA) and endoperoxides, according to Heath and Packer (1968) and modified by Hagege et al. (1990). MDA routinely used as an indicator of lipid peroxidation was extracted with 5% (w/v) trichloroacetic acid. A reaction with 2-thiobarbituric acid (TBA) was conducted at 95°C for 30 min. Then the samples were cooled to room temperature and specific absorbance was measured at 532 nm, but non-specific absorbance was recorded at 600 nm. The results were expressed as nmol MDA equivalents per 1 g of FW.

All experiments were repeated at least three times and all test samples were analyzed three to four times. The results presented are the mean ± SD.

Analyses of UV-absorbing compounds (UVAC)

Lentil roots were immersed in 80% (v/v) methanol in a ratio of 10 ml g^{-1} FW, and then incubated at 55°C for 30 min. Absorption spectra were obtained at room temperature using a Hitachi U-2001 spectrophotometer. A relative accumulation of PhC was determined by measuring the absorbance at 270 nm (peak absorption value) of each extract from full spectral analysis (Lois 1994; Białońska et al. 2007).

Determination of copper content in tissues

Copper content in the roots of seedlings was determined after tissue wet mineralization with HNO_3 and H_2SO_4 by flame atomic absorption spectrophotometry (FAAS; AAS-3 Zeiss; Posmyk et al. 2009). All analyses were triplicated and the results are mean ± SD.

Extraction of PhC from roots

Lentil roots were lyophilized and the dry material was ground in a coffee mill. PhC were extracted from the ground material using 80% (v/v) methanol at a solids-to-solvent ratio of 1:10 (w/v) (Amarowicz et al. 2005) in an ultrasonic water bath at 50°C for 5 min. Then, the sample was centrifuged and the supernatant was collected. The extraction was repeated at least twice and the supernatants were combined. The procedure was repeated using 80% (v/v) acetone. The organic solvent was evaporated off under vacuum at 40°C using a rotary evaporator. Residual water was removed from the extract by lyophilization.

Determination of total PhC

The content of total PhC in the extract was estimated using Folin-Ciocalteu's phenol reagent (Amarowicz et al. 2004). The results were calculated using a standard curve for (+)-catechin. All analyses were triplicated and the results are mean ± SD.

Separation of phenolic acids from crude extract

Phenolic acids were separated from the crude extract according to Weidner et al. (1999). Briefly, a portion of the extract was dissolved in 10 ml of 2 M NaOH and then hydrolyzed for 4 h at room temperature under a nitrogen atmosphere. After acidification to pH 2 using 6 M HCl, free phenolic acids and those released from phenolic esters were extracted five times into 1 ml of diethyl ether using a separatory funnel. The ether layer was evaporated to dryness with a rotavapor. The free phenolic acid residues and the phenolic acids released from esters were dissolved in 2 ml of methanol followed by filtration through a 0.45 μm membrane.

Acid hydrolysis of flavonoids in the crude extract

Acid hydrolysis of phenolic compounds from lentil crude extracts was carried out according to Crozier et al. (1997). Briefly, a portion of the extract was dissolved in 5 ml of 1.2 M HCl in 50% (v/v) aqueous methanol containing 0.2% (m/v) *tert*-butylhydroquinone. The solution was heated at 90°C for 2 h. After hydrolysis, the sample's volume was adjusted to 25 ml with distilled water.

HPLC analysis of phenolic acids and flavonoids

Phenolic acids were analysed using a Shimadzu HPLC system (Shizadzu Corp., Kyoto, Japan) comprising an LC-10AD pump, SCTL 10A system controller and SPD 10A photodiode array detector. Each sample was first filtered through a 0.45 μm nylon membrane and then injected onto a prepacked LiChrospher 100 RP-18 column (4 × 250 mm, 5 μm; Merck, Darmstad, Germany). The mobile phase consisted of water:acetonitrile:acetic acid (88:10:2; v/v/v), the flow rate was 1 ml min^{-1}, and detection of phenolic acids was monitored at 320 nm.

The same Shimadzu HPLC system was used for the analysis of flavonoids present in the raw extract and released after acid hydrolysis. Gradient elution of acetonitrile:water:acetic acid (5:93:2, v/v/v) [solvent A] and of acetonitrile:water:acetic acid (40:58:2, v/v/v) [solvent B], 0–50 min solvent B from 0 to 100% were used (Crozier et al. 1997). The separation of compounds was monitored at 350 nm. After HPLC analysis the content of phenolic

acids and kaemferol in the injected sample was calculated from the plot of peak area versus external standard concentration. All analyses were triplicated and the results are mean ± SD.

Statistical analyses

The results were analysed using Statgraphics Plus v. 4.1. First, one-way ANOVA was performed on each organ in order to test whether there were differences between treatments ($P < 0.05$). Then, Tukey's test was carried out to compare the treatments pairwise.

Results

Effect of Cu^{2+} on copper accumulation and root growth

The control seedling roots of both lentil cultivars contained about 1.026 μg Cu g^{-1} DW. After 3 days of cultivation in the presence of Cu^{2+}, the content of this metal in the roots increased markedly, but was comparable in both lentil cultivars (Fig. 1). However, 5-day exposition to this metal caused higher accumulation of Cu in the roots of cv. Tina, while in cv. Krak no significant changes were observed (Fig. 1).

The roots of the control 3- and 5-day-old cv. Krak seedlings were longer than those of cv. Tina. During 3 days of Cu^{2+} treatment, there were no significant differences in root elongation of both cultivars. Retarding effects of roots elongation occurred after 5 days of metal treatment. The length of the roots was reduced by about 60 and 80% in cvs. Krak and Tina, respectively, in comparison to the control (Table 1). Moreover, Cu^{2+} treatment of both cultivars caused a decrease in the fresh as well as dry weight of the roots (Table 1).

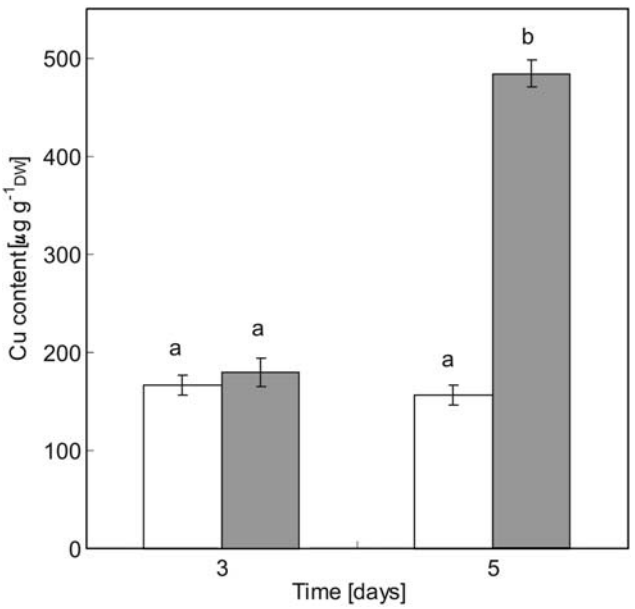

Fig. 1 Copper accumulation in the roots of lentil seedlings cvs. Krak (*white rectangles*) and Tina (*black rectangles*) exposed for 3 and 5 days in the presence of Cu^{2+} at 0.5 mM. The content of Cu in the control roots of both cultivars was similar, 1.027 μM g^{-1} DW. *Values* for the seedlings at the same age followed by the *same letter* do not differ significantly at $P < 0.05$ by Tukey's pairwise comparisons

Effect of Cu^{2+} on lipid peroxidation

Lipid peroxidation levels in the roots of lentil seedlings, evaluated as thiobarbituric acid-reactive substances (TBARS), are presented in Fig. 2a, b. In the control, the lipid peroxidation level was much higher in cv. Tina than in cv. Krak. Cu^{2+} increased the TBARS levels in both tested cultivars, but the effect was significantly more pronounced in cv. Tina (Fig. 2). In cv. Krak, the level of TBARS on the 3rd and 5th day of cultivation in the presence of Cu^{2+} was the same, twice as high as in the control seedlings (Fig. 2a), while in cv. Tina the metal caused a twofold and threefold increase in TBARS levels after 3- and 5-day treatments, respectively, in comparison to the control roots (Fig. 2b).

Effect of Cu^{2+} on PhC content

The content of the total PhC in the control roots in cv. Tina was significantly higher than in cv. Krak. In cv. Krak, Cu^{2+} treatment increased the total PhC content by 8 and 31% above the control and in cv. Tina Cu^{2+} decreased it to 80 and 69% after 3 and 5 days, respectively (Table 2).

In the roots of lentil seedlings, the compounds that absorb UV light accumulated at 270 nm (UVAC; Fig. 3a, b). In the control roots of cv. Tina the UVAC content was much higher than in cv. Krak (Fig. 3a, b). Cu^{+2} treatment stimulated their accumulation in both investigated cultivars, but after 5 days their increase in cv. Krak roots was 140% and in cv. Tina only about 5% above the control (Table 2; Fig. 3a, b).

To investigate the nature of these UVAC, we collected methanol extracts from the non-treated (control) and Cu^{2+}-treated roots and analysed them by HPLC (Figs. 4, 5). Similar HPLC profiles for the phenolic acid and flavonoids were obtained for both cultivars. The HPLC results, free phenolic acids and those released from phenolic esters indicated the presence of hydroxycinnamic acid derivatives in the lentil roots mainly consisting of ferulic (FA) and *p*-coumaric (*p*-CA) acids (Fig. 4) and the flavonol, kaempferol (Kam; Fig. 5).

In the control roots, the contents of FA and Kam were higher in cv. Tina than in cv. Krak (Table 3). Cultivation in the presence of Cu^{2+} resulted in an increase in FA

Table 1 Effect of Cu^{2+} on growth parameters in the roots of seedlings from two lentil cultivars in the control (0) and Cu^{2+} (0.5 mM) supplemented media

Cu^{2+} (mM)	cv. Krak		cv. Tina	
	0	0.5	0	0.5
Seedlings of 3 days old				
Root length (cm)	3.5 ± 0.26 b	1.71 ± 0.19 a	2.71 ± 0.21 b	1.32 ± 0.34 a
FW (mg)	30.4 ± 0.6 b	19.1 ± 0.8 a	14.8 ± 1.2 b	10.3 ± 0.3 a
DW (mg)	2.1 ± 0.1 b	1.7 ± 0.1 a	1.25 ± 0.0 b	0.8 ± 0.3 a
Seedlings of 5 days old				
Root length (cm)	6.8 ± 0.5 b	2.69 ± 0.53 a	5.23 ± 0.45 b	1.01 ± 0.16 a
FW (mg)	35.2 ± 0.8 b	20.3 ± 0.3 a	30.6 ± 0.6 b	16.7 ± 0.3 a
DW (mg)	2.8 ± 0.1 b	2.1 ± 0.4 a	2.2 ± 0.5 b	1.2 ± 0.0 a

Mean values of two experiments with two to four parallel analyses ± SE. Values in each vertical column followed by the same letter do not differ significantly at $P < 0.05$ by Tukey's pairwise comparisons

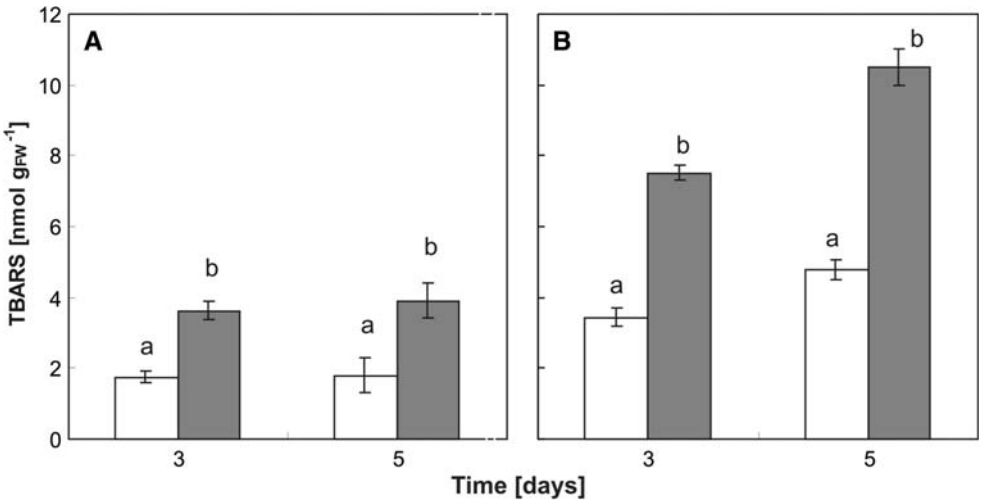

Fig. 2 Effect of Cu^{2+} at 0.5 mM on TBARS levels in the roots of 3- and 5-day-old lentil seedlings cv. Krak (**a**, *white rectangles*) and cv. Tina (**b**, *black rectangles*). Means of six measurements ± SD. *Values* for seedlings at the same age followed by the *same letter* do not differ significantly at $P < 0.05$ by Tukey's pairwise comparisons

Table 2 Effect of Cu^{2+} (0.5 mM) on total phenolic compounds (PhC) and UVAC in roots of 3- and 5-day-old lentil cultivars of Krak and Tina

Concentration of Cu^{2+} (mM)	cv. Krak5		cv. Tina	
	0	0.5	0	0.5
Seedlings of 3 days old				
UVAC, % of control	100	164	100	146
PhC (mg g^{-1} DW)	1.93 ± 0.06 a	2.08 ± 0.08 a	3.08 ± 0.09 b	2.48 ± 0.07 a
Control%	100	108	100	80
Seedlings of 3 days old				
UVAC, % of control	100	240	100	105
PhC (mg g^{-1} DW)	1.52 ± 0.05 a	1.99 ± 0.06 b	4.09 ± 0.12 b	2.81 ± 0.08 a
Control%	100	131	100	69

Values in each vertical column followed by the same letter do not differ significantly at $P < 0.05$ by Tukey's pairwise comparisons

(156–140% of the control) and *p*-CA (106–124% of the control) in the cv. Krak roots. In the case of cv. Tina seedlings, during the first period (3 days of stress), a 30% decrease in FA and *p*-CA was noted. Only after 5 days did the amounts of both increase. As Kam levels are concerned, little changes in both cultivars were observed (Table 3).

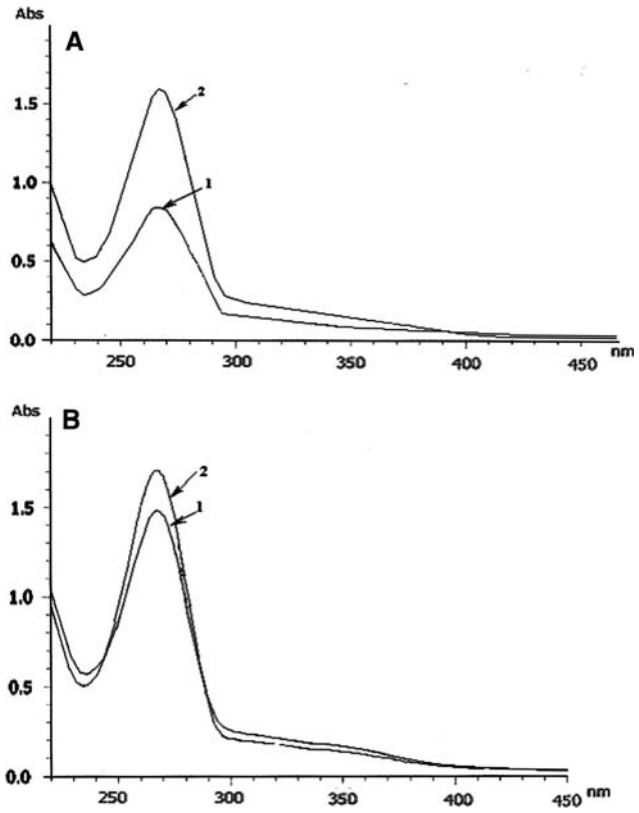

Fig. 3 Absorbance spectra of root ethanol extracts in the control (*1*) and Cu^{2+}-treated (*2*) roots of the seedlings after 5 days, cvs. Krak (**a**) and Tina (**b**)

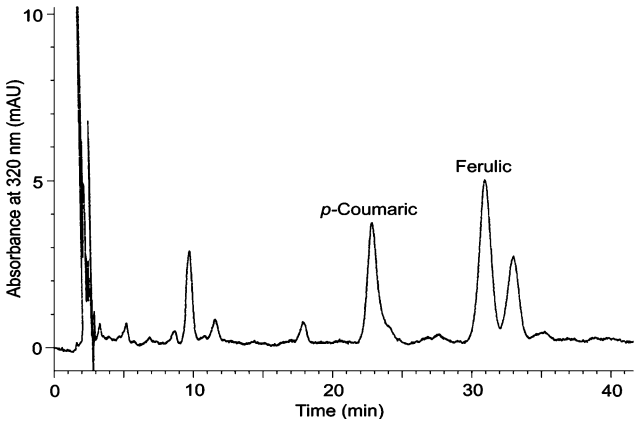

Fig. 4 A typical HPLC profile of soluble phenolic acids extracted from 5-day-old roots of lentil seedlings cv. Krak collected from plants grown in Cu^{2+} (0.5 mM) presence. The roots of cv. Tina grown under $+Cu^{2+}$ and $-Cu^{2+}$ conditions showed similar HPLC profiles of phenolic acids

Discussion

The results described in this paper indicate that Cu^{2+} at the high concentration of 0.5 mM in both lentil cultivars inhibited the elongation of roots as well as the production of their dry and fresh weight (Table 1). A significant

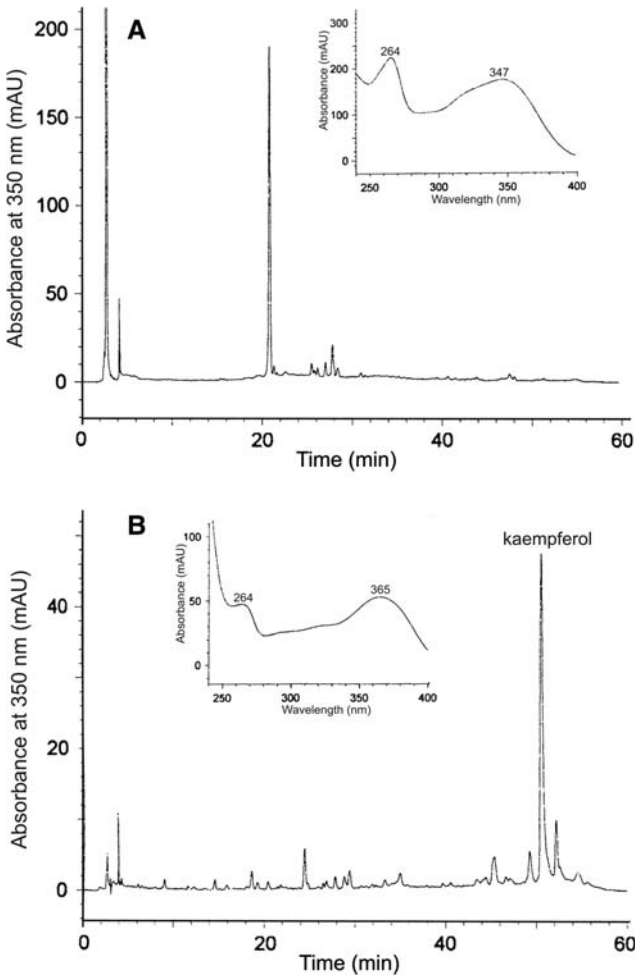

Fig. 5 A typical HPLC profile of flavonoids of root extracts from 5-day-old lentil seedlings cv. Krak collected from plants grown in Cu^{2+} (0.5 mM) presence. The roots of cv. Tina grown under $+Cu^{2+}$ and $-Cu^{2+}$ conditions showed similar HPLC profiles of flavonoids. **a** Sample of crude extract. **b** Sample after acid hydrolysis

inhibition of root elongation and biomass accumulation in different plant species treated with Cu^{2+} was observed (Ahsan et al. 2007; Kováčik et al. 2008). In both lentil cultivars, the growth of roots was more sensitive to Cu^{2+} than the growth of epicotyls, and it is worth noting that the roots accumulated more Cu (data not shown). Different studies demonstrated that a large portion of Cu absorbed by plants was retained in the roots (Panou-Filotheou and Bosabalidis 2004; Kováčik et al. 2008). This may be a consequence of the preferential accumulation of this metal in these organs and its low mobility inside the plant (Fargašova 2001). After 3 days of Cu^{2+} treatment, the accumulation of Cu was enhanced in both cultivars; however, after 5 days the content of this metal further increased in cv. Tina but did not change in cv. Krak (Fig. 1). It is known that plants possess several strategies to tolerate heavy metals in their environment such as their exclusion,

Table 3 Contents of soluble ferulic acid (FA), *p*-coumaric acid (*p*-CA) and kaempferol (Kam) in μg g^{-1} DW in the roots of seedlings of two lentil cultivars in the control (0) and Cu^{2+} (0.5 mM)-supplemented media

$CuSO_4$ (mM)	cv. Krak			cv. Tina		
	FA	*p*-CA	Kam	FA	*p*-CA	Kam
Seedlings of 3 days old						
0	33.3 ± 1.5 a	32.3 ± 1.5 a	1,018.3 ± 85 a	49 ± 2.0 b	32.7 ± 2.0 a	1,220 ± 35 a
	100%	100%	100%	100%	100%	100%
0.5	52 ± 2.0 b	34.3 ± 1.5 a	1,096.7 ± 73.5 a	35.3 ± 1.5a	24 ± 1.0 b	1,424.3 ± 30 b
	156%	106%	108%	72%	73%	117%
Seedlings of 5 days old						
0	34.7 ± 1.5 a	28.3 ± 1.5 a	1,161.7 ± 36 a	46 ± 2.0 a	29 ± 1.0 a	1,572.7 ± 34.5 b
	100%	100%	100%	100%	100%	100%
0.5	48.7 ± 1.5 b	35.3 ± 1.5 b	1,144 ± 39.6 a	55 ± 2.0 b	38 ± 2.0 b	1,412 ± 27.8 a
	140%	124%	98%	120%	131%	90%

Mean values of two experiments with three parallel analyses ± SE. Values in each vertical column followed by the same letter do not differ significantly at $P < 0.05$ by Turkey's pairwaise comparisons

restricting heavy metal uptake, sequestration of metal in organs and organelles and/or phytochelatin binding (Maksymiec 1997). Our data may justify a hypothesis that cv. Krak employs the strategy of metal exclusion and tolerance to high Cu^{2+} concentration in the roots.

The exposure of the seedlings to excess of Cu^{2+} led to higher enhancement of both metal accumulation and TBARS level in the roots of cv. Tina (Fig. 2b) in comparison to cv. Krak (Fig. 2a). These observations indicate that the excess of Cu inside the roots of cv. Tina induced oxidative stress by generating a high level of ROS, which are regarded as initiators of peroxidative cell damage (Garcia et al. 1999; Gaetke and Chow 2003; Tewari et al. 2006).

The maximum absorption peak of methanol extracts, most probably representatives of PhC from the roots of lentil seedlings, was observed at 270 nm (UVAC). Higher amounts of UVAC were observed in cv. Krak exposed to Cu^{+2} than in cv. Tina (Fig. 3a, b). On the other hand, UV spectra depicted in Fig. 3 can evidence the presence of other PhC, which were not determined by the applied gradient system of the HPLC method. It is also worth noting that methanol extraction does not differ among individual compounds and may also include several other compounds besides flavonoids and hydroxycinnamic acids (Białońska et al. 2007).

In Cu^{2+}-treated roots of cv. Krak the amount of total PhC increased, while in cv. Tina it decreased in comparison to the control (Table 2). Various heavy metals induced accumulation of PhC (Babu et al. 2003; Ali et al. 2006; Górecka et al. 2007), but in same cases reduction in PhC was noted in plants exposed to metal (Loponen et al. 2001; Caldwell 2002; Roitto et al. 2005; Jahangir et al. 2008). Our observations may suggest that the induction of PhC in Cu^{2+}-treated roots of cv. Krak plays an important role in the protection of lentil roots against the metal, similar as in root suspension cultures of *Panax ginseng* C.A. Meyer (Ali et al. 2006), *Lemna gibba* (Babu et al. 2003), primary leaves of runner bean (*Phaseolus coccineus* L.; Skórzyńska-Polit et al. 2004), in plant material regenerated from embryos obtained in anther culture of *Daucus carota* (Górecka et al. 2007), *Raphanus sativus* (Sgherri et al. 2002) and chamomile (*Matricaria chamomilla* L.) plants (Kováčik et al. 2008).

HPLC analyses of UVAC revealed mainly the presence of derivatives of hydroxycinnamic acids, FA and *p*-CA (Fig. 4; Table 3), and flavonol - Kam (Fig. 5; Table 3). As indicated by our results, the levels of FA and *p*-CA increased after 3 and 5 days of stress in cv. Krak, while in cv. Tina the content of these compounds was lower after 3 days and increased after a 5-day stress. Therefore, it can be assumed that these compounds are involved in an antioxidative system as suggested by Sgherri et al. (2002) and can be lignin precursors, which reduce the toxic effects of metals and their uptake into root tissue (Chen et al. 2002). It is known that PhC, such as FA and *p*-CA, are active antioxidants (Rice-Evans et al. 1997), but can also cause suppression of lipid peroxidation (Terao et al. 1994). Under metal stress, plants produce secondary metabolites, whose concentration increases with growing metal concentrations up to a certain point, beyond which a decrease in secondary metabolites level can be observed (Jahangir et al. 2008). Thus, while the lower PhC levels in the Cu^{2+}-treated roots of cv. Tina are probably due to stress, their higher levels in cv. Krak can still be a response to ROS signaling.

The difference between the content of total PhC (Table 2) and the content of individual PhC determined

with HPLC method (Table 3) is not an error. The ability of individual phenolics to reduce Folin-Ciocalteu's phenol reagent varies greatly (e.g. molar absorptivity per reactive group ranges from 0.1 for flavone to 20.2 for malvin) (Singleton et al. 1999) and can cause overestimation of the results obtained.

As observed in this study, Kam level hardly changed under metal stress (Table 3). It is evident that the response of various plant species to different kinds of stress can differ considerably in terms of flavonoid synthesis. Changes in flavonoid levels may depend on the age of plants, organs, stressors, time of stress and varieties of plants (Caldwell 2002; Winkel-Shirley 2002; Skórzyńska-Polit et al. 2004; Jahangir et al. 2008). It is known that flavonoid aglycons are stronger antioxidants than flavonoid glycosides (Rice-Evans et al. 1997; Lim et al. 2008). Greater changes in the content of phenolic acids than of Kam may suggest that the former play a more important role in protecting lentil roots against toxic Cu^{2+} concentration. These results may also suggest that another acclimation process, besides PhC synthesis, can participate in the acclimation of lentil to Cu^{2+} stress (Babu et al. 2003; Sgherri et al. 2002; Maksymiec 2007; Posmyk et al. 2009).

In conclusion, the present study shows that the high concentration of Cu^{2+} caused a growth inhibition in both cultivars. This metal induced oxidative damage as indicated by the accumulation of lipid peroxidation products. Decline in total PhC and an increase in lipid peroxidation in cv. Tina may indicate that the level of oxidative stress generated by the presence of the excessive concentration of Cu^{2+} was so high that the capacity of antioxidative systems was exceeded. However, though the high concentration of Cu^{2+} stimulated PhC in cv. Krak, it was not sufficient to counteract the amount of ROS generated by metal presence. Thus, it seems that similarly as in light-grown *Lemna gibba* (Babu et al. 2003) also in the roots of dark-grown seedlings in the presence of Cu^{2+}, ROS can be a common signal inducing PhC accumulation.

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