DOI: 10.2478/10004-1254-61-2010-2059

Scientific Paper

EFFECT OF COPPER ON THE TOXICITY AND GENOTOXICITY OF CADMIUM IN DUCKWEED (*LEMNA MINOR* L.)

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Received in July 2010
Accepted in August 2010

We investigated interactions between copper (in the concentrations of 2.5 μmol L^{-1} and 5 μmol L^{-1}) and cadmium (5 μmol L^{-1}) in common duckweed (*Lemna minor* L.) by exposing it to either metal or to their combinations for four or seven days. Their uptake increased with time, but it was lower in plants treated with combinations of metals than in plants treated with either metal given alone. In separate treatments, either metal increased malondialdehyde (MDA) level and catalase and peroxidase activity. Both induced DNA damage, but copper did it only after 7 days of treatment. On day 4, the combination of cadmium and 5 μmol L^{-1} copper additionally increased MDA as well as catalase and peroxidase activity. In contrast, on day 7, MDA dropped in plants treated with combinations of metals, and especially with 2.5 μmol L^{-1} copper plus cadmium. In these plants, catalase activity was higher than in copper treated plants. Peroxidase activity increased after treatment with cadmium and 2.5 μmol L^{-1} copper but decreased in plants treated with cadmium and 5 μmol L^{-1} copper. Compared to copper alone, combinations of metals enhanced DNA damage after 4 days of treatment but it dropped on day 7. In conclusion, either metal given alone was toxic/genotoxic and caused oxidative stress. On day 4 of combined treatment, the higher copper concentration was more toxic than either metal alone. In contrast, on day 7 of combined treatment, the lower copper concentration showed lower oxidative and DNA damage. These complex interactions can not be explained by simple antagonism and/or synergism. Further studies should go in that direction.

KEY WORDS: *aquatic plants, DNA damage, ecotoxicological testing, heavy metals, oxidative stress*

Heavy metals are very persistent natural or anthropogenic pollutants, especially in the aquatic environment. Most are toxic and indirectly genotoxic to various organisms. The biological effects of individual metals are more or less known, but even though combinations of heavy metals are common in nature their combined effects are still to be thoroughly investigated (1, 2). Metals in mixtures may act independently or interact to produce additive, synergistic, or antagonistic effects, which depends on their concentrations and factors such as temperature, pH, and light (2). In general, these interaction effects do not fit the toxicological profiles of individual metals making a combination.

Cadmium (Cd) is one of the most toxic metals, and is also genotoxic for most plant and animal organisms (1-4). It is released into the environment through industrial wastewater and other waste. It accumulates in the soil, water, and sediment. Cd induces tumours in exposed experimental animals and human cell lines (4). However, the molecular mechanism of Cd genotoxicity is still unclear. Cd genotoxicity could

be induced directly by its interaction with DNA or by inhibition of essential mismatch repair, both causing genetic instability (4). Moreover, Cd can induce oxidative stress and can damage biological macromolecules such as lipids, proteins and DNA through reactive oxygen species (ROS) (5). In plants, Cd induces DNA damage (6) directly or indirectly by inducing oxidative stress (7). Cd can also affect the photosynthetic apparatus (8, 9), as well as the respiratory and nitrogen metabolism, which results in growth retardation, leaf chlorosis, water and nutrient imbalances, and the production of ROS (3). Unlike Cd, copper (Cu) at low concentrations is essential for numerous physiological processes. Its concentration in soil solution for optimal growth of most plant species is in the range of 10^{-3} μmol L^{-1} to 1 μmol L^{-1} (10). However at high concentrations it inhibits root growth, induces chlorosis, necrosis, and leaf discoloration, and disturbs very important cell processes (11). Moreover, it can damage cell membranes and induce lipid peroxidation since Cu, like other heavy metals, induces oxidative stress (12, 13).

Chemical and physical analytical methods are useful for detecting the presence and the concentration of chemicals in the environment, but these methods do not include biological component. Plants are routinely used for biological monitoring of xenobiotics (14, 15). Among them, macrophytes are very suitable for toxicology testing, family *Lemnaceae* in particular. The advantage of aquatic macrophytes over terrestrial plants is that macrophytes are exposed not only through the root, but also through the leaf. Aquatic macrophytes duckweeds have commonly been used to test the toxicity of heavy metals (16) because they are very easy to culture in the laboratory. Their growth rate is high and so is sensitivity to different pollutants. Aquatic plants are of special environmental concern, as they biofilter toxic elements (17). In recent years, interest has been focused on using aquatic plants, such as *Lemna minor*, *Microspora*, and *Pistia stratiotes*, as a promising approach to take up heavy metals from water body (18, 19). *Lemna minor* L., also known as common duckweed, is often used in ecotoxicological testing as a representative of higher aquatic plants (20).

So far, only a few studies have investigated combined effects of metals on plant species (1). The aim of our study was to investigate whether Cu, as an essential element, interacted with the toxicity and genotoxicity of Cd, a non-essential metal, in aquatic plant *Lemna minor* L. (duckweed).

MATERIALS AND METHODS

Plant material and culture conditions

Lemna minor L. stock culture was maintained on a Pirson and Seidel nutrient medium (21). For the experiment the plants were grown in sterilised Steinberg medium (22) at (24±1) °C, with a 16-hour light (40 μE m^{-2} s^{-1}) and 8-hour dark cycle.

Cd and Cu treatment

Approximately 10 colonies of healthy duckweed were transferred into a 300 mL Erlenmeyer flask containing 100 mL of Steinberg nutrient solution with Cd (in the form of $CdCl_2$; Fluka, Switzerland) at the concentration of 5.0 μmol L^{-1} and/or Cu (in the form of $CuCl_2$; Kemika, Croatia) at 2.5 μmol L^{-1} or 5.0 μmol L^{-1}. Plants grown on Steinberg medium alone were used as control. Metal content, lipid peroxidation, carbonyl content, comet assay, catalase (CAT) and peroxidase (POD) activities in duckweed fronds were determined on days 4 and 7 of exposure.

Determination of Cu and Cd content

Plants were oven-dried at 80 °C for 24 h until a constant weight was achieved. Plant tissue was then microwave digested in two steps. The first digestion step was digestion in 10 mL of concentrated HNO_3 (16 mmol L^{-1}; Kemika, Croatia) at 70 °C for 5 min, then at 130 °C for another 5 min and finally at 150 °C for 4 min. The second step was digestion in 1 mL of H_2O_2 (Kemika, Croatia) at 85 °C for 5 min and then at 130 °C for 4 min. After cooling, the samples were diluted with 1 % (v/v) HNO_3 up to the total volume of 50 mL. Cadmium and copper were analysed using inductively coupled plasma-Optical Emission Spectroscopy (ICP-OES, IRIS INTREPID II XSP 9597 – Thermo Elemental, USA) according to the HRN EN ISO 11885:1998 standard. The results were processed using TEVA software /firmware version 1.5.0/1.5.0 (Thermo Elemental Validated Analysis, Thermo Electron Corporation, USA). Metal concentrations were calculated according to the calibration curve obtained with a set of standards of known concentrations (Merck, Germany). For cadmium we used a lower concentration range of 1 μg L^{-1} to 50 μg L^{-1}, and for copper a higher concentration range of 50 μg L^{-1} to 5,000 μg L^{-1}. Detection limits for Cd and Cu were 0.5 μg kg^{-1} and 10 μg kg^{-1}, respectively. The limit of quantification (LOQ) was <1 μg kg^{-1} and <20 μg kg^{-1}, respectively.

Malondialdehyde and carbonyl content

Lipid peroxidation was determined by measuring the amount of malondialdehyde (MDA), a product of lipid peroxidation, according to a modified method of Heath and Packer (23). Treated plants (50 mg fresh weight) were homogenised in 1 mL of 0.25 % (w/v) 2-thiobarbituric acid (TBA, Sigma, Germany) in 10 % trichloroacetic acid (TCA, Kemika, Croatia), and incubated at 95 °C for 30 min. The tubes were then transferred into an ice bath and centrifuged at 15,000 *g* and +4 °C for 10 min. The absorbance of the supernatant was recorded at 532 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. For a blank we used 0.25 % TBA in 10 % TCA. The content of MDA was expressed as micromol per gram of fresh weight (μmol g^{-1}) using an extinction coefficient of 155 L $mmol^{-1}$ cm^{-1}.

For carbonyl quantification, the reaction with 2,4-dinitrophenylhydrazine (DNPH, Aldrich, Germany) was used basically as described by Levine et al. (24). Fresh plant tissue (80 mg) was homogenised in 1 mL of 10 mmol L^{-1} potassium phosphate buffer (pH 7.4), which contained 1 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA, Merck, Germany) and polyvinylpyrrolidone (PVP, Sigma, Germany). After centrifugation at 20,000 *g* and +4 °C for 20 min, the supernatants (200 μL) were combined with 300 μL of 10 mmol L^{-1} DNPH in 2 mol L^{-1} HCl (Kemika, Croatia). After 1-hour incubation at room temperature, the proteins were precipitated with 500 μL of cold 10 % (w/v) TCA. Samples were cooled to -20 °C and then centrifuged at 12,000 *g* and +4 °C for 10 min. The pellets were washed three times with 500 μL of ethanol/ethylacetate (1:1, v/v) to remove excess reagent. The precipitated proteins were finally dissolved in 6 mol L^{-1} urea in 20 mmol L^{-1} potassium phosphate buffer (pH 2.4) in an ultrasonic bath. Absorbance was measured at 370 nm. Protein recovery was estimated for each sample by measuring the absorbance at 280 nm. Carbonyl content was calculated using a molar absorption coefficient for aliphatic hydrazones of 22 L $mmol^{-1}$ cm^{-1} and expressed as micromol per milligram of proteins (μmol mg^{-1}).

Comet assay

For the genotoxicity assessment, we used an alkaline version of the cellular comet assay following the protocol by Gichner et al. (6) with a slight modification (10 min denaturation, 10 min electrophoresis at 0.72 V cm^{-1} and 300 mA). Duckweed leaves were placed in a 60 mm diameter Petri dish containing 200 mL of ice-cold 400 mmol L^{-1} Tris/HCl buffer (Kemika, Croatia; pH 7.5) and cut into small pieces with a sterile razor blade to isolate the nuclei. Fifty randomly chosen nuclei per treatment from two independent experiments were analysed under a fluorescent microscope (Zeiss Axioplane) equipped with an excitation filter BP 520/09 nm and a barrier filter of 610 nm. A computerised image analysis system (Komet version 5, Kinetic Imaging Ltd., Liverpool, UK) was used to measure percentage of tail DNA (% tDNA).

Enzyme activity assays

Plants (80 mg) were homogenised in 1 mL of cold 50 mmol L^{-1} potassium phosphate buffer (Kemika, Croatia; pH 7.0), containing 0.1 mmol L^{-1} EDTA and PVP. The homogenate was centrifuged at 20,000 *g* and +4 °C for 30 min and the supernatant was used for the CAT and POD enzyme assays.

CAT (EC 1.11.1.6) activity was evaluated spectrophotometrically (25) by measuring the decrease in H_2O_2 absorbance at 240 nm (ε=36 L $mmol^{-1}$ cm^{-1}) every 10 s for 2 min. The reaction mixture consisted of 50 mmol L^{-1} of potassium phosphate buffer (pH 7.0), 10 mmol L^{-1} of H_2O_2, and 50 μL of enzyme extract. CAT activity was expressed as micromol of decomposed H_2O_2 per minute per milligram of proteins (μmol min^{-1} mg^{-1}). For CAT in-gel detection, the gels were incubated in distilled water for 45 min and then in H_2O_2 solution (0.003 %, v/v) for 10 min. The gels were then washed in distilled water and stained in a 1:1 mixture of 2 % (w/v) $FeCl_3$ and 2 % (w/v) $K_3Fe(CN)_6$ (Kemika, Croatia) for 10 min (26).

POD activity was determined spectrophotometrically by measuring the increase in absorbance at 470 nm (ε=26.6 L $mmol^{-1}$ cm^{-1}) every 15 s for 2.5 min. The reaction mixture consisted of 50 mmol L^{-1} of potassium phosphate buffer (pH 7.0), 18 mmol L^{-1} of guaiacol (Sigma, Germany), 5 mmol L^{-1} of H_2O_2 (27), and 50 μL of enzyme extract. POD activity was expressed as micromol of formed tetraguiacol per minute per milligram of proteins (μmol min^{-1} mg^{-1}). For POD in-gel detection, the gels were equilibrated with 50 mmol L^{-1} potassium phosphate buffer (pH 7.0) for 30 min and then incubated in 50 mmol L^{-1} potassium phosphate buffer (pH 7.0) containing 20 mmol L^{-1} pyrogallol and 4 mmol L^{-1} H_2O_2 until brown bands appeared (27).

Soluble protein content was determined according to Bradford (28), using bovine albumin serum as a standard.

Statistical analysis

The results of each assay were compared by analysis of variance (ANOVA), Newman-Keuls test using the STATISTICA 8.0 (Stat Soft Inc., USA) software package. Differences between corresponding controls and exposed samples were considered statistically significant at $P<0.05$. Each data point is the average of six replicates unless stated otherwise.

RESULTS

Cd and Cu content

On day 4 of the experiment, Cd content in plants exposed to 5 μmol L^{-1} Cd alone was significantly higher than in plants exposed to either combination of Cd and Cu (Table 1). The combination of Cd and Cu in the higher concentration (5.0 μmol L^{-1}) significantly decreased Cd content in comparison to the combination the lower Cu concentration (2.5 μmol L^{-1}) and even more distinctly to Cd alone. Cu was the highest in duckweed exposed to Cu alone in the higher concentration (5.0 μmol L^{-1}), while in combination with Cd, it was significantly lower. The lowest Cu content was measured in plants exposed to 2.5 μmol L^{-1} Cu, alone or in combination with Cd.

On day 7, Cd content showed the same behaviour as on day 4. The highest Cu content was observed in plants exposed to 5 μmol L^{-1} Cu alone, while it was significantly lower in plants exposed to the combination of 5 μmol L^{-1} Cu and Cd. Cu content in plants exposed to 2.5 μmol L^{-1} Cu alone was significantly lower than in plants exposed to 5 μmol L^{-1} Cu either alone or in combination with Cd. However, in comparison to combined exposure to 2.5 μmol L^{-1} Cu and Cd it was significantly higher (Table 1).

In plants not exposed to Cd (control and Cu-treated plants), Cd content was below the instrument LOQ (<0.001 μg g^{-1}). The same goes for Cu content in plants not exposed to Cu (control and Cd-treated plants, <0.02 μg g^{-1}).

Lipid peroxidation and protein oxidation

MDA level was significantly higher in all treated plants than in control plants on both days 4 and 7 of the experiment. On day 4, the highest MDA was observed in plants exposed to a combination of 5 μmol L^{-1} Cu and Cd. Very high MDA levels were also measured in plants treated with 2.5 μmol L^{-1} Cu alone, but were similar between other treatments (Figure 1).

On day 7, the highest MDA was observed in plants exposed to Cu alone in either concentration. MDA levels dropped in plants exposed to to the combination of 5 μmol L^{-1} Cu and Cd, and even more to the combination of 2.5 μmol L^{-1} Cu and 5 μmol L^{-1} Cd, yet these values were still significantly higher than in control plants (Figure 1).

Table 1 *Cadmium and copper content in Lemna minor L. on days 4 and 7 of exposure to Cd (5.0 μmol L^{-1}) and Cu (2.5 μmol L^{-1} and 5.0 μmol L^{-1}) and their combinations. In some samples, Cd and Cu were below the instrument's detection below the instrument's limit of quantification (LOQ; <0.001 μg g^{-1} and <0.02 μg g^{-1}, respectively). The values are expressed as means ±SE of at least four replicates from two individual experiments ± SE. Significant differences (P<0.05, Newman-Keuls test) between days 4 and 7 are marked with different letters.*

	Treatments	Cd / μg g^{-1} dry weight	Cu / μg g^{-1} dry weight
Day 4	Control	<LOQ[a]	<LOQ[a]
	Cd 5.0 μmol L^{-1}	834.32[d] ± 33.66	<LOQ[a]
	Cu 2.5 μmol L^{-1}	<LOQ[a]	244.71[b] ± 25.53
	Cu 5.0 μmol L^{-1}	<LOQ[a]	363.81[d] ± 14.71
	Cu 2.5 μmol L^{-1} + Cd 5.0 μmol L^{-1}	536.1[c] ± 65.86	232.22[b] ± 17.71
	Cu 5.0 μmol L^{-1} + Cd 5.0 μmol L^{-1}	431.54[b] ± 11.65	310.9[c] ± 9.76
Day 7	Control	<LOQ[a]	<LOQ[a]
	Cd 5.0 μmol L^{-1}	1055.64[d] ± 37.94	<LOQ[a]
	Cu 2.5 μmol L^{-1}	<LOQ[a]	342.91[c] ± 36.36
	Cu 5.0 μmol L^{-1}	<LOQ[a]	480.98[e] ± 27.82
	Cu 2.5 μmol L^{-1} + Cd 5.0 μmol L^{-1}	808.84[c] ± 52.44	264.51[b] ± 12.11
	Cu 5.0 μmol L^{-1} + Cd 5.0 μmol L^{-1}	698.81[b] ± 60.3	405.87[d] ± 18.43

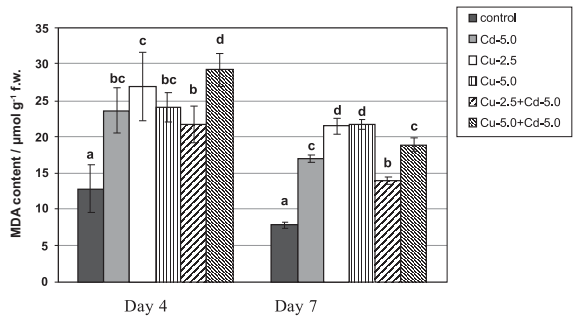

Figure 1 *Differences in MDA levels on days 4 and 7 of the experiment. Values are expressed as means±SE, based on six replicates. Different column letters indicate significant differences at P<0.05 according to the Newman-Keuls test.*

On day 4, carbonyl level, indicating oxidative damage to proteins, was higher in the exposed plants than in controls, but not significantly.

On day 7, however, it increased significantly in plants treated with Cu alone and with the combination of 5 μmol L^{-1} Cu and Cd (Figure 2).

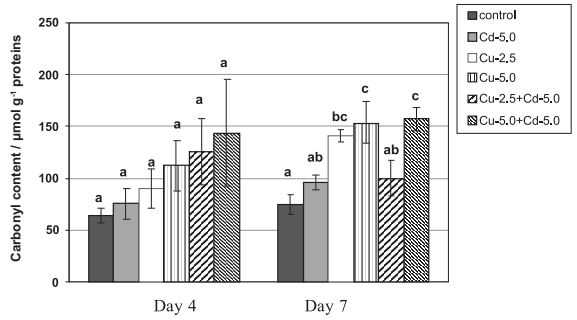

Figure 2 *Differences in protein carbonyl levels on days 4 and 7 of the experiment. Values are expressed as means±SE, based on six replicates. Different column letters indicate significant differences at P<0.05 according to the Newman-Keuls test.*

Effect on DNA

On day 4, the percentage of tail DNA showed a significant DNA damage in duckweed exposed to Cd alone or to both combinations with Cu. Cu alone, however, did not show any genotoxic effect.

On day 7, DNA damage in plants exposed to Cu alone soared significantly at either concentration. Interestingly, in plants exposed to Cu and Cd combinations the level of DNA damage dropped significantly to values similar to control (Figure 3).

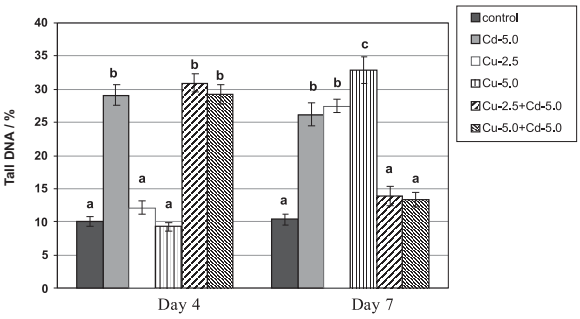

Figure 3 *Differences in percentage of tail DNA on days 4 and 7 of the experiment. Values are expressed as means±SE, based on six replicates. Different column letters indicate significant differences at P<0.05 according to the Newman-Keuls test.*

Effect on antioxidant enzyme activity

On day 4, CAT significantly rose in plants treated with Cd alone in respect to control and Cu-exposed plants. However, absolutely the highest CAT activity was recorded in plants exposed to either combination (Figure 4A).

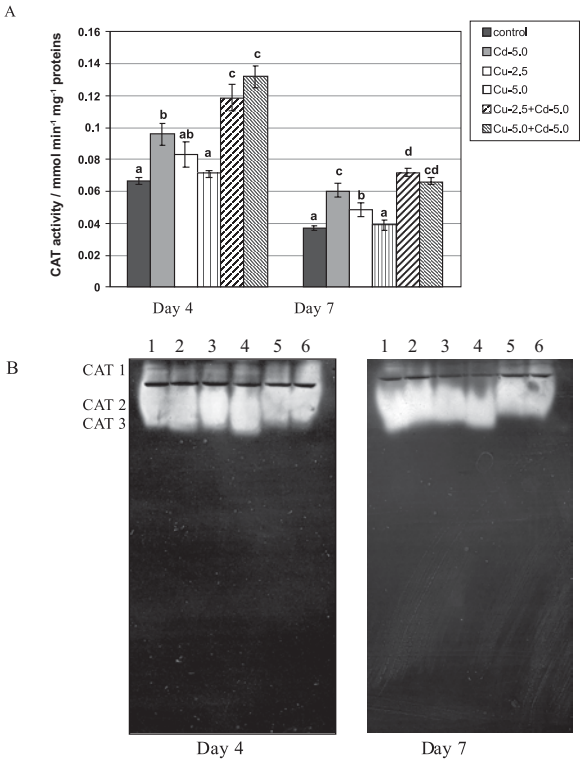

Figure 4 *Differences in A) CAT activity on days 4 and 7 of the experiment. Values are expressed as means±SE, based on six replicates. Different column letters indicate significant differences at P<0.05 B) Isoenzyme pattern of CAT on days 4 and 7. Equal amounts of proteins (100 μg) were loaded on the gel. 1 - control; 2 - Cd 5.0 μmol L^{-1}; 3 - Cu 2.5 μmol L^{-1}; 4 - Cu 5.0 μmol L^{-1}; 5 - Cd 5.0 μmol L^{-1} + Cu 2.5 μmol L^{-1}; 6 - Cd 5.0 μmol L^{-1} + Cu 5.0 μmol L^{-1}*

Similar results were recorded on day 7 (Figure 4A).

Electrophoresis revealed three CAT isoenzymes, which were marked as CAT1-CAT3, according to their rising mobility (Figure 4B). Isoforms CAT1 and CAT3 were common to control plants and plants treated with either Cd alone or Cu alone in both concentrations. Plants exposed to the combination of Cd and Cu revealed additional CAT2 isoenzyme and lacked isoform CAT3.

POD activity on day 4 significantly increased in plants treated with Cd alone and Cu alone at the lower concentration in comparison to control plants and plants treated with Cd and Cu at the lower concentration (Figure 5A). The highest POD activity was observed in plants treated with Cd and Cu at the higher concentration.

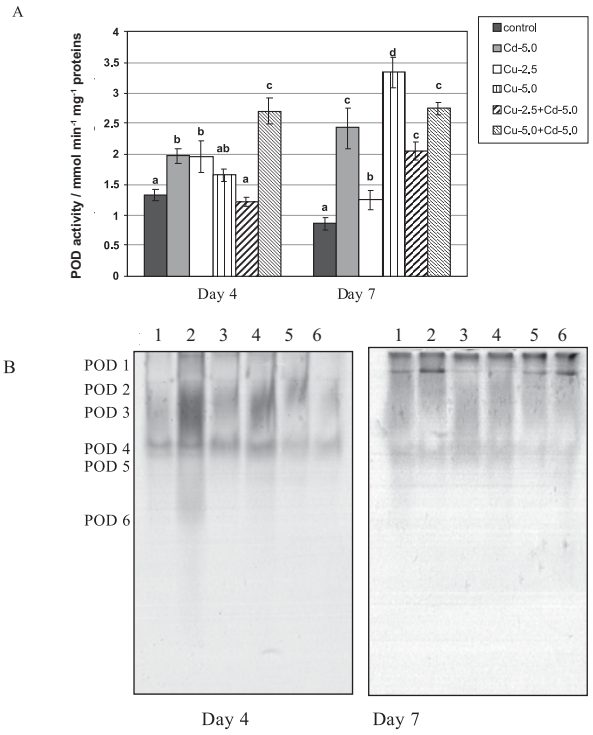

Figure 5 *Differences in A) POD activity on days 4 and 7 of the experiment. Values are expressed as means±SE, based on six replicates. Different column letters indicate significant differences at P<0.05 according to the Newman-Keuls test. B) Isoenzyme pattern of POD on days 4 and 7. Equal amounts of proteins (100 µg) were loaded on the gel. 1 - control; 2 - Cd 5.0 µmol L^{-1}; 3 - Cu 2.5 µmol L^{-1}; 4 - Cu 5.0 µmol L^{-1}; 5 - Cd 5.0 µmol L^{-1} + Cu 2.5 µmol L^{-1}; 6 - Cd 5.0 µmol L^{-1} + Cu 5.0 µmol L^{-1}*

On day 7, all treated plants exhibited higher POD activity than control plants; the highest POD activity was observed with Cu at the higher concentration (5.0 µmol L^{-1}). Cd and both Cd/Cu combinations produced similar POD activities, which were higher than the one with 2.5 µmol L^{-1} Cu alone (Figure 5A).

On day 4, six POD isoforms were detected and marked as POD1-POD6 according to their rising mobility (Figure 5B). Treatment with Cd yielded the highest POD2 and POD3 in-gel activity. Isoforms POD1-POD5 were present in all samples, while POD6 was appeared with Cd treatment only.

On day 7, isoform POD1 gave a stronger signal in all samples than on day 4 , while POD2 was more pronounced in Cd and both Cd/Cu combinations. Isoform POD6 was not found in samples treated with Cd alone.

DISSCUSION

Cadmium and copper are important environmental pollutants, especially to aquatic ecosystems. Although they differ in biological significance, higher concentrations of these metals are toxic and disturb plant metabolism. The choice of Cd concentration in this study relies on findings from a previous study (29), while the choice of Cu concentrations is based on preliminary experiments yielding reduced growth, but without chlorosis.

Our study has shown that both metals tend to accumulate with time, but also that in combination, they affect each other's uptake. The higher Cu concentration of 5.0 µmol L^{-1} was more effective in inhibiting Cd uptake than the lower Cu concentration of 2.5 µmol L^{-1}. Generally, plants do not absolutely discriminate between important micronutrients and nonessential metals, and different metals may share the same transporters across the cell membrane (30).

Given the complexity of the transmembrane transport of metals, further studies at molecular level are needed to understand the mechanism underlying this phenomenon (30). Semsari et al. (31) have shown that *Lemna gibba* removes Cd from the growth medium in linear and concentration-dependent manner, but with Zn and Cu the removal is biphasic, with a quick and slow phase. Kwan and Smith (32) have shown that Ca affects Cd accumulation in *Lemna minor.* A study with other macrophytes has also suggested that micronutrients are taken up by plants faster than non-essential heavy metals (33).

On the other hand, metal combinations supplemented to growth media can interact and affect each other's accumulation (34), but interaction does not necessarily

coincide with the bioaccumulation pattern (35). This is why we analysed various oxidative stress parameters, to get a better understanding of Cu-Cd interaction.

Lipid peroxidation, which diminishes the integrity of cells and organelle membranes, is one of the most significant effects of heavy metals in plants (12, 36). Increased MDA level is considered a general indicator of oxidative stress (37). Increased MDA has already been found in *L. minor* and other species after exposure to Cd (38, 29) or Cu (13). Our results have shown significantly higher MDA in all exposed plants as early as day 4. The highest MDA content found in plants treated with a combination of Cd and 5 μmol L^{-1} Cu may point to a synergistic effect. On day 7, however, the highest MDA level was found in plants treated with Cu alone, while it dropped in plants treated with metal combinations. This points to a reduction in oxidative stress in plants treated with the metal combinations, which could correlate with higher CAT activity.

In plants exposed to heavy metals, protein levels are often lower in consequence of lower Mg and K uptake, nutrients important for protein synthesis (38). Oxidative damage to proteins was measured using carbonyl as a biomarker. When oxidised, proteins change their native conformation as well as activity level (39). This could mean that proteins are more susceptible to oxidative damage by Cu than by Cd, probably due to direct ROS production in the presence of Cu (10).

Comet assay showed a significant increase in DNA damage in Cd alone-treated plants throughout the experiment. Cu alone was genotoxic only on day 7. Interestingly, Cu combined with Cd showed the opposite behaviour; on day 4, DNA damage was high, only to plunge on day 7. Unfortunately, the comet assay, being a quantitative method, does not shed any light on the mechanism underlying this behaviour or metal genotoxicity as such. Our lipid peroxidation and carbonyl level findings suggest that metal damage DNA indirectly, that is, through oxidative stress. The same was observed by Gichner et al. (6) and Valverde et al. (40). This is supported by a drop in DNA damage in plants exposed to Cu/Cd combinations on day 7, which correlates with lower MDA level (Figure 1). The drop may also be attributed to DNA repair (41).

H_2O_2-scavenging enzymes CAT and POD have an important role in plant response to oxidative stress. Catalase is located in peroxisomes and its very high reaction rate turns it into an effective cellular sink for H_2O_2, despite its poor affinity for the molecule (42). Guaiacol peroxidase is located in cytosol, vacuole, cell wall, and extracellular space. Having a considerably higher affinity for H_2O_2 than catalase, POD requires a reductant, a substrate to be oxidised during H_2O_2 scavenging (43). Both CAT and a variety of peroxidases seem to have an important role in controlling H_2O_2. The enzymes are activated in response to elevated ROS as part of plant defence mechanism, although decline or inhibition of their activity has been also noticed under severe stress (29). In some plants, Cd and Cu are known to increase CAT and POD activity (29, 36, 44). In our study, CAT showed similar activity pattern on days 4 and 7. Cd alone caused a significant rise in CAT activity in comparison to control and Cu-treated plants. Previous studies in duckweeds have also indicated concentration-dependent changes in CAT activity after treatment with Cd or Cu (16, 38). In our study, both Cu/Cd combinations provoked the highest CAT activity, regardless of exposure time. This suggests that metal combinations are more stressful to plants than individual metals. This is further confirmed by the presence of isoenzyme CAT2 and absence of CAT3 in plants treated with metal combinations. Unlike CAT isoenzymes, changes in the number of POD isoenzymes were observed only in Cd-treated plants.

Cu and Cd are known to generate ROS in different manner, which could explain the differences between their effects. Cu is a redox-active metal, which catalyses hydroxyl radical production via Haber-Weis reaction, while Cd indirectly increases ROS load by binding to functional protein group, which eventually disrupts protein structure and inhibits protein activity. Yeh et al. (45) have shown that differences between Cu and Cd in generating ROS may produce different intracellular heavy metal stress signalling networks. Tkalec et al. (29) have also proposed that H_2O_2 signal-transducing molecule may be responsible for enhanced CAT and POD activities in *L. minor* exposed to Cd for a long time.

CONCLUSION

In this study, Cd and Cu, when in combination, reduced each other's uptake. Both metals were toxic/genotoxic to duckweed and caused oxidative stress when added to medium alone, but in combination they showed a more complex response that can not be explained by simple antagonism and/or synergism. Further studies should go in that direction.

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Sažetak

UČINAK BAKRA NA TOKSIČNOST I GENOTOKSIČNOST KADMIJA U VODENOJ LEĆI (*LEMNA MINOR* L.)

U svrhu istraživanja interakcija između bakra kao esencijalnog elementa te kadmija kao neesencijalnog i toksičnog metala, vodenu leću *Lemna minor* L. uzgajali smo na podlogama s kadmijem (5 μmol L^{-1}) odnosno s bakrom (2,5 μmol L^{-1} i 5 μmol L^{-1}) te s njihovim kombinacijama. Unos metala u biljke povećavao se s trajanjem pokusa, a kod kombinacije metala u biljkama je izmjerena niža količina kadmija nego u onima uzgajanima samo na kadmiju. U biljkama tretiranim pojedinačnim metalom došlo je do povećanja sadržaja malondialdehida (MDA) te aktivnosti katalaze i peroksidaze u odnosu na kontrolne biljke. Također, primijećeno je oštećenje DNA iako kod bakra tek sedmog dana tretmana. Količina MDA i aktivnost obaju enzima dodatno se povećala na tretmanu kombinacijom kadmija i bakra (5 μmol L^{-1}) nakon četvrtog dana pokusa, dok se količina MDA smanjila nakon sedmog dana kod kombinacije kadmija i 2,5 μmol L^{-1} bakra. U tim biljkama primijećena je i veća aktivnost katalaze, dok je aktivnost peroksidaze porasla na tretmanu kadmijem i 2,5 μmol L^{-1} bakrom, ali se smanjila na tretmanu kadmijem i 5 μmol L^{-1} bakrom. Oštećenje DNA koje je bilo veće kod kombinacije metala nakon četvrtog dana, osobito u usporedbi sa samim bakrom, smanjilo se nakon sedmog dana pokusa. Iz ovih rezultata može se zaključiti da su oba metala u istraživanim koncentracijama toksična i genotoksična za vodenu leću i da uzrokuju oksidacijski stres. Kadmij u kombinaciji s bakrom više koncentracije bio je toksičniji od pojedinačnih metala nakon četvrtog dana pokusa, dok su u biljaka tretiranih kombinacijom kadmija i bakra niže koncentracije toksični učinci bili manji. Budući da su primijećene interakcije vrlo kompleksne i ne uključuju samo antagonizam odnosno sinergizam potrebna su daljnja istraživanja.

KLJUČNE RIJEČI: *ekotoksikološka analiza, oksidacijski stres, oštećenje DNA, teški metali, vodene biljke*

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