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ASSESSMENT OF THE PHYSIOLOGICAL CONDITION OF *SALVINIA NATANS* L. EXPOSED TO COPPER(II) IONS

Salvinia natans L. is a known bioaccumulator of metal ions from the aquatic environment. Exposure of plants to prolonged impact of toxins along with a large dose of the metal affects the physiological condition of the plants. The changes occurring in the plants in the phytoremediation process in contaminated medium culture of 5, 10, 15 and 20 mg Cu/dm^3 show two basic physiological parameters: the assimilation pigment content (chlorophyll a and b) responsible for the production of energy in the process of photosynthesis, and the total protein level as the main constituent of the plant. Conducted studies confirmed a negative effect of high concentrations of copper(II) on *Salvinia natans* L. plants which in these conditions are capable to bioaccumulate metal from each contaminated culture medium.

1. INTRODUCTION

There is a great interest in using plants capable of accumulating large amounts of metals in tissues to purify contaminated aquatic ecosystems. The process of phytoremediation is based on the natural ability to retrieve, collect and biodegrade toxins within the plant tissue. It is used in the treatment of shallow water reservoirs with low contamination excess levels and in the source of contamination (the in-situ method). The pace of the process is closely associated with the selection of the species of plants and their growth. Large doses of pollutants are potentially dangerous for plants, causing serious damage and decay [1]. The use of native plant species in the process of phytoremediation is valid due to their tolerance to the existing climatic conditions. The plants' condition significantly influences the performance of the purification of water environment and the efficiency of the phytoremediation process. In the Odra valley there are sites occupied by the species of *Salvinia natans* which has confirmed the ability to bioaccumulate metal and non-metal ions from polluted waters [2–12]. *Salvinia* sp. cumulate

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90% of copper ions from solutions of low concentrations (up to 1.0 mg Cu/dm^3), as well as from solutions containing much more copper [2–6].

Basic parameters enabling to assess the physiological condition of the plant after the process of phytoremediation of the polluted aquatic environment are the total protein level and content of assimilation pigments in plants. Copper in the concentration ranging from 1.0 to 3.0 mg/dm^3 causes inhibition of growth of *Salvinia minima* but it does not stop it entirely [13]. However, there is little information on the reaction of the plants to the stress stemming from the contact with metal ions.

Bizzo et al. [14] investigated the influence of Cu in various concentrations (0.01, 0.1, 1 and 10 mM) on the development of *Salvinia auriculata* Ablu., in a short-time exposure of plants (2 days). The results have shown high tolerance of *Salvinia auriculata* Ablu. to solutions containing up to 1 mM of copper. The content of chlorophyll a decreased with the increase of metal in the solution, contrary do chlorophyll b. Similar changes of the content of chlorophyll a and b in *Lemna gibba* L. were recorded by Hegazy et al. [15] during the investigation of the influence of ions of heavy metals from the medium contaminated with copper and zinc on plants. The chlorophyll a to chlorophyll b ratio was changed due to the degradation of assimilation pigments. Dhir et al. [16] observed the decrease of the photosystem II activity in the *Salvinia natans* L. culture exposed to stress caused by the presence of 35 mg Cu/dm^3. After 48 h a 4-fold increase of the photosystem I activity under heavy metal stress (889 μmoles of O_2 consumed mg chl^{-1}h^{-1} was recorded in comparison to control (216,8 μmoles of O_2 consumed per 1 mg of chlorophyll per 1 h). The contents of the assimilation pigments were as follows: 152 μg of chlorophyll a/g of fresh matter (control: 261 μg of chlorophyll a/g of fresh matter), 173 μg of chlorophyll b/g of fresh matter (control: 180 μg of chlorophyll b/g of fresh matter) and 335 μg of total chlorophyll/g of fresh matter (control: 418 μg of total chlorophyll/g of fresh matter). The overall content of chlorophyll decreased also after 6 days of the exposition of *Salvinia natans* Kurth. [17] due to the effect of ions of copper, cadmium and zinc in the multimetallic medium (10 mg/dm^3 of each metal ion in the solution). The research confirmed lower sensibility of the assimilation pigments in *Salvinia natans* Kunth. compared to *Lemna minor* L., *Pistia stratiotes* L. and *Eichhornia crassipes* Mart. Moreover, changes were observed with regard to the total protein level. Stress caused by the presence of three heavy metals in the medium resulted in the decrease of the total protein level in *Salvinia natans* Kurth. from ca. 40 mg of protein/g of fresh matter (control) to ca. 15 mg of protein/g of fresh matter, and in the case of *Lemna minor* L., from 50 mg of protein/g of fresh matter (control) to 40 mg of protein/g of fresh matter after 6 days of the experiment.

Sensitivity to copper(II) ions of *Salvinia natans* L. was investigated using high doses of metal in aqueous solutions (5, 10, 15 and 20 mg Cu/dm^3) for a period of 14 days. Two basic parameters: the total protein level and contents of chlorophyll a and chlorophyll b were measured in order to examine the impact of copper ions accumulated in the tissues of plants on the physiological processes in *Salvinia natans* L.

2. MATERIALS AND METHODS

Native species of the plant (*Salvinia natans* L.) derived from the commercial plant breeding were selected for the experiment. The selected plants with good individual condition had long roots, irregularly distributed along the stem, thus increasing the surface area of contact with the medium culture. It could result in better uptake of substances contained in the medium culture. The breeding plants did not produce sporokarpium. The mature individuals formed lateral growths of smaller leaves with less intense color. Adaptation period of plants was introduced in order to allow them to acclimatize to the optimized laboratory conditions, similar to the conditions present in the natural environment in Poland. The plant breeding was carried out in the following conditions: 22±1 °C, humidity of 30±2% and the temperature of water 20±1 °C, using the Hoagland medium culture of the following composition: KNO_3 – 1.02 g/dm^3, $Ca(NO_3)_2{\times}4H_2O$ – 0.71 g/dm^3, $NH_4H_2PO_4$ – 0.23 g/dm^3, $MgSO_4{\times}7H_2O$ – 0.49 g/dm^3, $MnCl_2{\times}4H_2O$ – 1.81 mg/dm^3, H_3BO_3 – 2.86 mg/dm^3, $CuSO_4{\times}5H_2O$ – 0.08 mg/dm^3, $ZnSO_4{\times}7H_2O$ – 0.22 mg/dm^3, MoO_3 – 0.09 mg/dm^3, $FeSO_4{\times}7H_2O$ (0,5%) – 0.60 mg/dm^3 [18]. After a one month adaptation period, the plants were sieved to separate reactors. Young *Salvinia natans* L. plants were collected for the experiment. The Hoagland medium culture was contaminated with the $CuSO4{\times}5H_2O$. pH of pure Hoagland medium culture and copper(II) contaminated solutions was 4.5–4.8. The inoculation of plant organisms based on wet biomass was introduced into a separate reactor (the stationary culture), containing 5, 10, 15 and 20 mg Cu/dm^3, and pure Hoagland medium culture (control). The phytoremediation process was carried out for 14 days for each copper contaminated culture and control. The experiment was performed in triplicate. The plants recovered in their entirety from the respective reactor were analysed in the process of biochemical assay in duplicate. Simultaneously, at 24 h intervals, the plants were observed in order to enable the assessment of the toxic effect of copper on their morphology. During the phytoremediation of water contaminated with copper, the contents of chlorophyll a and b and the total protein level were measured.

Fresh biomass was homogenized in 90% acetone solution using an IKA Ultra-Turrax dispenser (6000 rpm for 5 min). The extraction of the assimilation pigments involved in the process of photosynthesis lasted for ca. 22 h at 2–8 °C [19].

Determination of the total protein level was carried out in fresh plant matter hydrolyzates using Lowry's method [20]. Preparation procedure was similar to that employed for the photosynthesis pigments, except for the hydrolyzate for determination of protein, where 1 M NaOH was used. Determination of total protein level was performed in the filtrates after the denaturation of protein at 100 °C for 10 min.

The filtrates of the extracts and hydrolyzates were analyzed by the spectrophotometry method with a T80+ UV/VIS spectrophotometer, PG Instruments Ltd. Absorbances were measured at the wavelength of 663 nm for chlorophyll a, 645 nm for chlorophyll b, and

750 nm for total protein. The content of assimilation pigments and total protein level were determined from reference curves.

The analyses were performed in the certified Toxicology and Environmental Research Laboratory of the Institute of Environment Protection Engineering, Wrocław University of Technology.

3. RESULTS AND DISCUSSION

Copper(II) ions are bound by proteins, affecting the physiological development of the plant. The average total protein level in the control sample was 21.44 mg of total protein/g of fresh matter (Fig. 1). The fluctuations in the total protein level in comparison to day 0 (21.96 mg of total protein/g of fresh matter) are small, in particular, up to the 10th day of the culture. The decrease in total protein level during the second week of the experiment did not exceed 9%. On the 14th day 20.02 mg of total protein/g of fresh matter was obtained (Fig. 1).

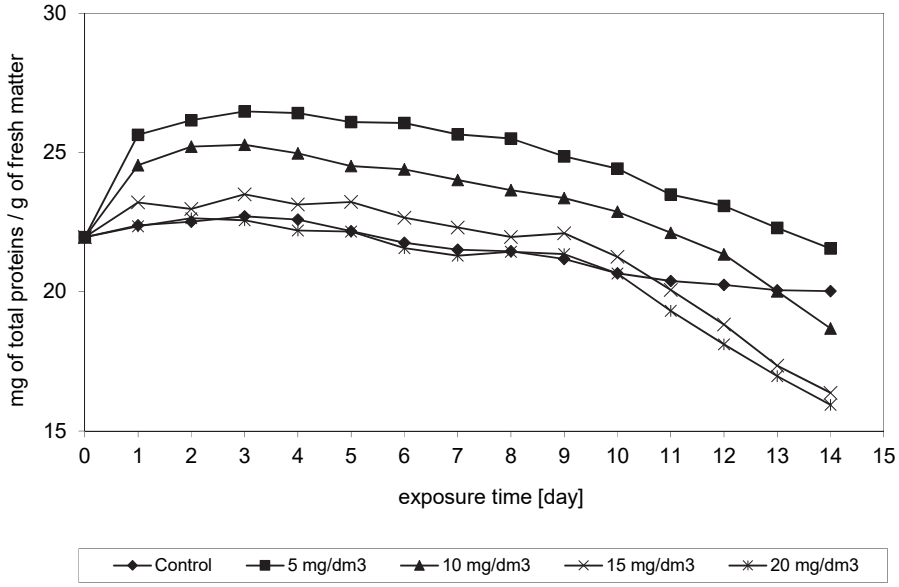

Fig. 1. Time dependence of the total protein content in plants

Natural ability of *Salvinia natans* L. to accumulate copper(II) ions may suggest increased total protein level in the plant observed in Fig. 1. The largest increase in total protein level in the biomass was observed in the first three days of the experiment. In the breeding contaminated with 5 mg Cu/dm^3, the maximum amount of 26.50 mg of total protein/g of fresh matter was observed on the 3rd da), which is a 21% increase in

the total protein. The amount of total protein in cultures of 5 and 10 mg Cu/dm^3 significantly exceeds the total protein level in the control (Figs. 1 and 2).

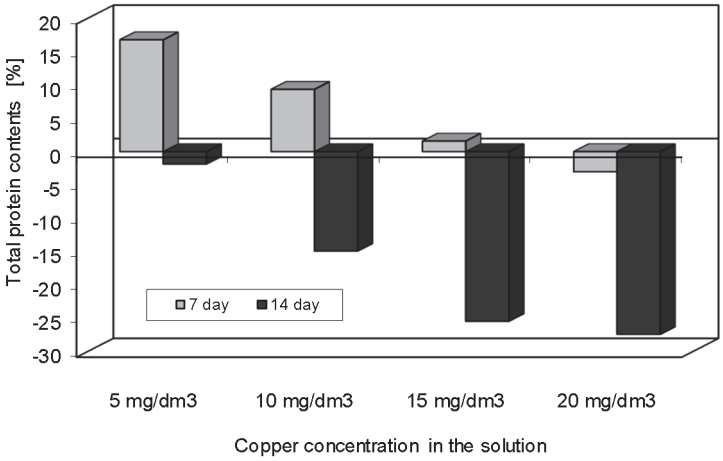

Fig. 2. Percentage change in total plant protein content

On the 7th and 14th day, 25.64 and 21.55 mg of total protein/g of fresh matter were obtained for 5 mg Cu/dm^3, respectively (Fig. 1) which corresponded to 17% increase on the 7th day and 2% decrease on the 14th day relative to control on day 0 (Fig. 2). For the 10 mg Cu/dm^3 breeding, the total protein level on respective experimental days was 24.01 and 18.68 mg of total protein/g of fresh matter, a 9% protein increase on the 7th day and 15% decrease on the 14th day was observed. For higher metal concentrations in the medium culture (15 and 20 mg Cu/dm^3), the total protein level in plants is close to the amount of protein in the control, particularly on the 7th day of the cultivation. For 15 mg Cu/dm^3, on the 7th and 14th day, the total protein level was 22.31 and 16.37 mg of total protein/g of fresh matter, which corresponds to 2% increase and 25% decrease on the respective days of cultivation. In the culture with 20 mg Cu/dm^3 on the same days, 21.29 and 15.94 mg of total protein/g of fresh matter was obtained (3 and 27% decrease).

The time of the plant's exposure to copper(II) ions in the medium culture above the 10th day significantly influenced the loss of total protein in the fresh matter which may be due to a general weakening of the physiological condition of the plants during the experiment at higher concentrations of Cu.

Buta et al. [17] conducted research with the use of *Salvinia natans* Kunth. (collected from the natural environment) in a solution contaminated with Cu, Cd, Zn ions, 10 mg/dm^3 each. On the 6th day, they received approx. 15 mg of total protein/g of fresh matter compared to control (40 mg of total protein/g of fresh matter), which corresponds to 38% decrease of total protein content. This may stem from the application of multimetallic medium in the plant culture, the type of plant and its origin. Our research with

the use of *Salvinia natans* L. (from an artificial culture) in the medium contaminated with 10 mg Cu/dm^3 (monometallic system) indicates a 12% increase of total protein content on the 6th day of the experiment compared to control. The protein content in the culture contaminated with copper amounted to 24.39 mg of total protein/g of fresh matter, whereas in control it equaled 21.76 mg of total protein/g of fresh matter. The decrease of total protein content was observed only in cultures with higher concentrations of copper in the medium.

The analysis indicates an increase in dry matter content in the subsequent days of the experiment in the medium cultures containing 5 and 10 mg Cu/dm^3. The increase was approximately by 20 and 12% with respect to the control. The dry matter increase in the control was 30% with respect to the initial matter (the 0 day). For 15 and 20 mg Cu/dm^3, a decrease in dry matter reaching almost 9% was observed on the 14th day for the highest concentrations of copper in the medium [2]. Copper in concentrations used in the experiment do not stimulate the increase of the biomass more than it does it in the control. In breeding with the highest concentrations of copper, the physiological condition of *Salvinia natans* L. might be affected by the duration of exposure to the metal, copper concentration in the medium culture as well as on the bioaccumulation of copper in plant tissues. Occasionally copper in low concentrations could stimulate plant's adaptation to selected environmental conditions. In this case, copper had negative effects on the growth of *Salvinia natans* L.

The analysis of copper content in biomass indicated an increase of metal content in plants in all medium cultures contaminated with copper(II) ions. On the 14th day of the experiment 7-, 18-, 23- and 40-fold increase in the metal content was obtained in the biomass of *Salvinia natans* L. for 5, 10, 15 and 20 mg Cu/dm^3 in the medium culture with respect to the control [2].

The analysis of the amount of the assimilation pigments responsible for the acquisition of energy by autotrophic organisms in fresh matter of the plant clearly shows the sensitivity of the body to high concentrations of copper in the medium culture. For all test concentrations of copper in the medium culture, the average assimilation pigments content in relation to the values obtained for the control were reduced (Fig. 3). The content of assimilation pigments in plants showed a significant reduction in the first days of their exposure to the set dose of copper.

In the control, the chlorophyll a content was maintained at a similar level (Fig. 3) and amounted to about 0.61 mg of chlorophyll a/g of fresh matter (0.63 mg of chlorophyll a/g of fresh matter on the 0 day). The decrease in the chlorophyll a content in the second week of the experiment was about 9.5% (0.57 mg chlorophyll a/g of fresh matter at the 14th day). For the copper concentration of 5 mg Cu/dm^3 in the medium culture clear decrease in the chlorophyll a content of about 28% compared to control was observed which corresponds to 0.44 mg chlorophyll/g of fresh matter on the 7th day of the experiment and 0.10 mg of chlorophyll/g of fresh matter on the 14th day (84% decrease in the chlorophyll a content compared to control) as shown in Figs. 3, 5. In the medium culture of 10 mg Cu/dm^3 the chlorophyll a content on the 7th and 14th day of the experiment

was 0.23 and 0.10 mg chlorophyll/g of fresh matter, what is the loss of chlorophyll a by 63 and 84% relative to control. Higher concentrations of copper in the medium (15 and 20 mg Cu/dm^3) clearly inhibit the synthesis of assimilation pigments what can be attributed to the toxicity of high doses of copper. For the 15 mg Cu/dm^3 concentration in the medium culture the chlorophyll a content on the 7th and 14th day of the experiment (Fig. 3 and 5) was 0.16 and 0.06 mg of chlorophyll/g of fresh matter (74 and 90% decrease compared to control) and for the 20 mg Cu/dm^3 concentration the chlorophyll a content represented 0.16 and 0.05 mg chlorophyll/g of fresh matter (loss of 74 and 92% compared to control).

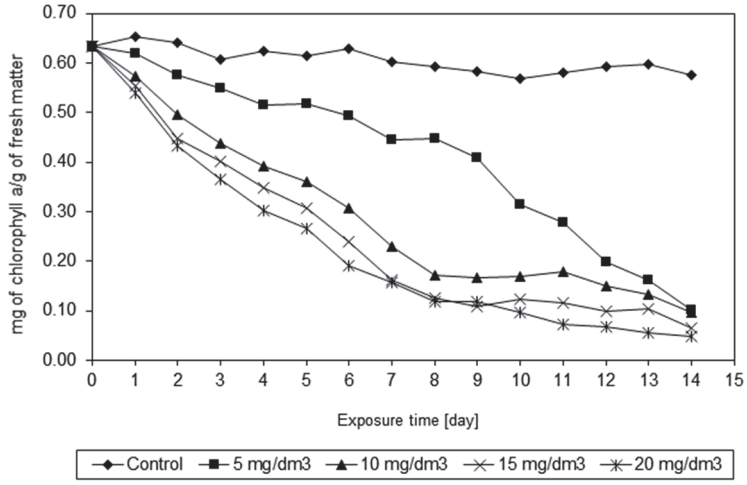

Fig. 3. Time dependence of the chlorophyll a content in plants

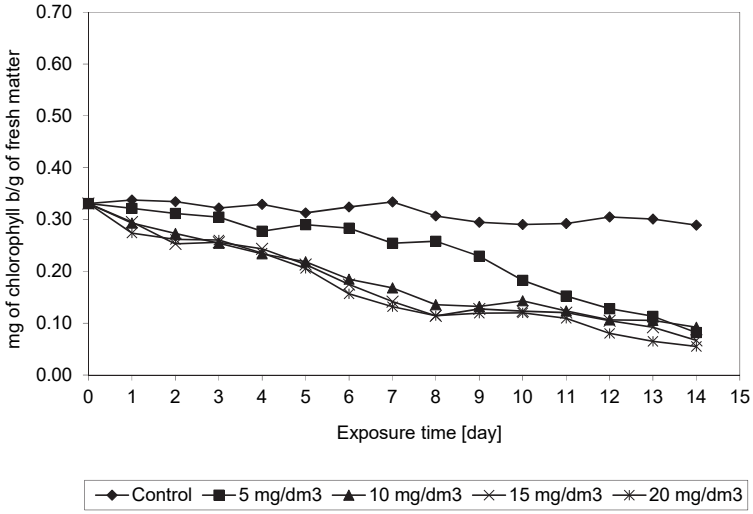

Fig. 4. Time dependence of the chlorophyll b content in plants

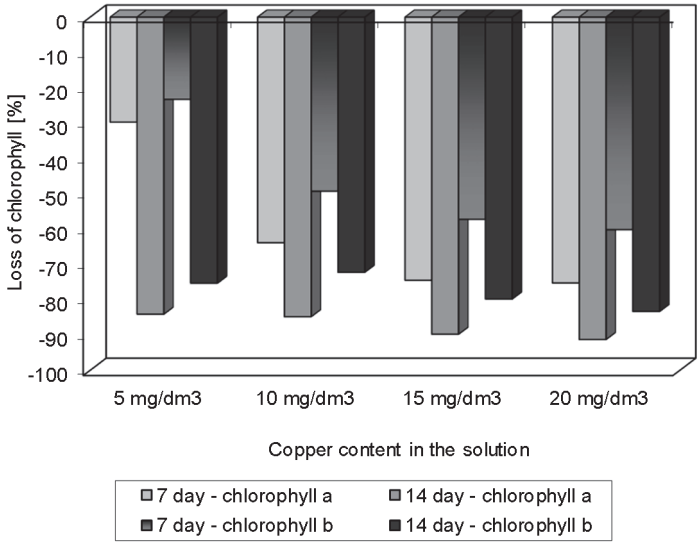

Fig. 5. Loss of chlorophyll a and chlorophyll b in plants

The chlorophyll b to chlorophyll a ratio in plants is 1:2. The chlorophyll b content in control was approximately 0.31 mg of chlorophyll/g of fresh matter while it was 0.33 mg chlorophyll/g of fresh matter on day 0 (Fig. 4). The chlorophyll b content in control on the 14th day was 0.29 mg chlorophyll/g of fresh matter (decrease by 12% compared to the 0 day).

The loss of chlorophyll b content on the 7th and 14th day of the phytoremediation process was less significant than in the case of chlorophyll a, as shown in Fig. 5. In the medium culture with 5 mg Cu/dm^3, the decrease in the chlorophyll b content was 19 and 74% on the 7th and 14th day of the experiment, corresponding to 0.25 and 0.08 mg of chlorophyll/g of fresh matter, respectively (Fig. 4). In other medium cultures with 10, 15, 20 mg Cu/dm^3 concentrations the chlorophyll b content in plants is similar in the subsequent days of culture (Fig. 4). On the 7th day of the experiment, the decrease of the chlorophyll b was 45, 55 and 58% which corresponds to 0.17, 0.14 and 0.13 mg of chlorophyll/g of fresh matter, respectively. The loss of chlorophyll b on the 14th day was 71, 77 and 81% (0.09, 0.07 and 0.06 mg of chlorophyll/g of fresh matter (Figs. 4, 5). The loss of the assimilation pigments (chlorophyll a and b) in the plants indicates that the excess of metal in the environment is the stress factor causing inhibition of the photosynthesis. The effect of copper toxicity could be observed in the changes in pigmentation, leading to plant fragmentation and the chlorosis.

Buta et al. [17] observed a decrease of chlorophyll a content in *Salvinia natans* Kurth. (originating from natural environment) in the breeding culture in the multimetallic medium containing Cu, Cd, Zn, 10 mg/dm^3 of each metal ion. After 6 days chlorophyll a content equaled 58% of the control. In the case of chlorophyll b, the amount of

this assimilation pigment practically remained unchanged (nearly 100%). Total chlorophyll content amounted to 72% of the initial value.

In our experiments with *Salvinia natans* L., on the 6th day of the breeding in the medium containing 10 mg Cu/dm^3 49% decrease of chlorophyll a content (306 μg of chlorophyll a/g of fresh matter) was observed (control – 628 μg of chlorophyll a/g of fresh matter). Chlorophyll b content was 185 μg of chlorophyll b/g of fresh matter, which is a 57% decrease with respect to control (324 μg of chlorophyll b/g of fresh matter). Total chlorophyll content on the 6th day amounted to 442,5 μg of total chlorophyll/g of fresh matter (51% of initial value).

Bizzo et al. [14] in experiments with *Salvinia auriculata* Ablu. cultures at lower concentrations of copper (0.01 and 0.1 mM Cu) after 2 days obtained 430.2 (79% of initial value) and 495.4 (91%) μg of chlorophyll a/g of fresh matter (control: 544.7 μg of chlorophyll a/g of fresh matter), 193.2 (94%) and 216.0 (105%) μg of chlorophyll b/g of fresh matter (control: 205.4 μg of chlorophyll b/g of fresh matter) and 632.4 (84%) and 711.3 (95%) μg of total chlorophyll/g of fresh matter (control: 750.2 μg of total chlorophyll/g of fresh matter).

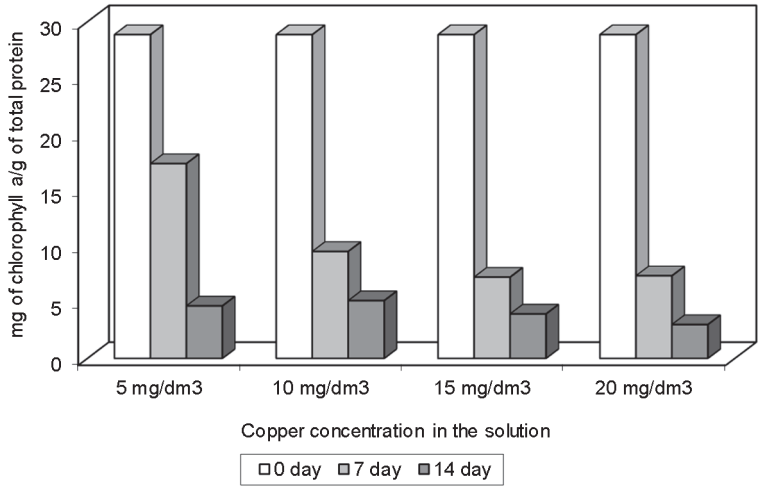

Fig. 6. Chlorophyll a content calculated for the total protein in plants

The results of our research indicate a decrease of the content of chlorophyll a and b on the 2nd day of the experiment in the media with the 5 and 10 mg Cu/dm^3. In the medium containing 5 mg Cu/dm^3 577 (90%) μg of chlorophyll a/g of fresh matter (control: 641 μg of chlorophyll a/g of fresh matter) and 312 (91%) μg of chlorophyll b/g of fresh matter (control: 334 μg chlorophyll b/g of fresh matter) were obtained, respectively. The total chlorophyll content in the medium with the 5 mg Cu/dm^3 amounted to

819.3 (95%) μg of total chlorophyll/g of fresh matter (control: 864.5 μg of total chlorophyll/g of fresh matter). In the medium with 10 mg Cu/dm^3 on the 2nd day of the experiment 496 (77%) μg of chlorophyll a/g of fresh matter (control: 641 μg of chlorophyll a/g of fresh matter) and 273 (82%) μg of chlorophyll b/g of fresh matter (control: 334 μg of chlorophyll b/g of fresh matter) were obtained, respectively. The total chlorophyll content amounted to 690.3 (80%) μg of total chlorophyll/g of fresh matter (control: 864.5 μg of total chlorophyll/g of fresh matter). The chlorophyll content calculated for 1 g of total protein, being the main building component of the plants, decreased (Fig. 6).

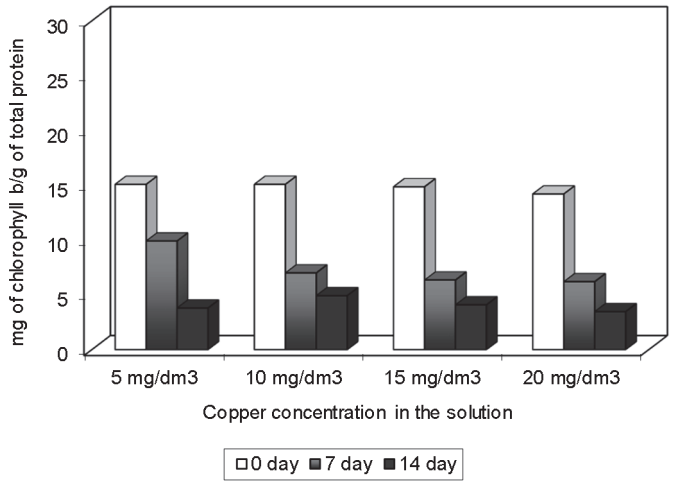

Fig. 7. Chlorophyll b content calculated for the total protein in plants

In the control, the content of the assimilation pigments was on average 28.29 mg of chlorophyll a/g of total protein and 14.80 mg of chlorophyll b/g of total protein. In all cultivations contaminated with copper(II) ions, a decrease in the assimilation pigments content calculated for the total protein was observed (Figs. 6 and 7).

In the cultivations containing 5 mg Cu/dm^3, the contents assimilation of pigments on the 7th and 14th day of the experiment were 17.36 and 4.67 mg of chlorophyll a/g of total protein and 9.90 and 3.79 mg of chlorophyll b/g of total protein, respectively. The decrease in chlorophyll was by 39% and in chlorophyll b by 33% on the 7th day, and 84 and 74% on the 14th day, respectively. For 10 mg Cu/dm^3 in the culture medium 5.15 and 9.53 mg of chlorophyll a/g of total protein (on the 7th and 14th day), as well as 6.99 and 4.92 mg of chlorophyll b/g of total protein was obtained. This corresponds to the decrease by 66 and 82% (chlorophyll a) and 53 and 67% (chlorophyll b). Similar assimilation pigments content calculated for the total protein was obtained in the cultivations containing 15 and 20 mg Cu/dm^3. On the 7th day of the experiment, in the breeding with the highest concentrations of copper in the medium culture approximately 7.0 mg of chlorophyll/g of total protein was observed (a decrease by 75% of chlorophyll a and 58%

of chlorophyll b). On the 14th day about 3.0 mg of chlorophyll/g of total protein was obtained (a decrease by 89% of chlorophyll a and 75% of chlorophyll b).

4. CONCLUSIONS

- The observation of plant morphology indicates changes in the pigmentation of plants and their fragmentation when extensively exposed to metal, particularly in cultures with copper of high concentrations in the medium culture (15 and 20 mg Cu/dm^3).
- The total protein level in plants in medium cultures with 5 and 10 mg Cu/dm^3 suggests a beneficial effect of copper(II) ions – an increase of total protein compared to the control. In cultures with 15 and 20 mg Cu/dm^3, a decrease in the total protein level in the last days of the experiment was observed (after 10 days).
- Copper(II) ions cause a decrease in chlorophyll a and b contents from the same beginning of the experiment in all cultures which proves a negative effect of copper in selected concentrations in the medium cultures.

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