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Original Research

The Effect of Gibberellic Acid (GA_3) on Growth, Metal Biosorption and Metabolism of the Green Algae *Chlorella vulgaris* (Chlorophyceae) Beijerinck Exposed to Cadmium and Lead Stress

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Abstract

The aim of this work was to show how gibberellic acid (GA_3) affects growth, metal biosorption, and the content of essential metabolites (cell number, proteins, monosaccharides, chlorophyll *a* and *b,* and total carotenoids) in unicellular green algae *Chlorella vulgaris* treated with lead (Pb) and cadmium (Cd) during 3 days of culture. Under the influence of 10^{-5} M GA_3, algal cells bioaccumulated and bioconcentrated toxic metals from nutrient medium in a dose- and exposure-dependent manner. Moreover, this phytohormone protected *C. vulgaris* against Pb and Cd stress at a range of low concentrations 10^{-7}-10^{-6} M inducing the increase in cell number and protein, photosynthetic pigment, and monosaccharide content in the culture. However, GA_3 was not able to minimize the harmful effects of the highest dose (10^{-4} M) of Cd and Pb, because growth inhibition and reduction in metabolite level was observed. The data suggest that GA_3 plays an important role in the growth and metabolism of microalgae *C. vulgaris* exposed to heavy metal stress and its adaptation ability to a low-level polluted aquatic environment.

Keywords: cadmium, cell number, chlorophylls, gibberellic acid (GA_3), lead, monosaccharides, proteins, total carotenoids

Introduction

Plants respond to many environmental factors such as water, mineral salts, heavy metals, carbon dioxide, light, oxygen, and temperature. Survival under these conditions may depend on the plant's ability to generate and transmit signals that adjust the metabolism accordingly [1]. Therefore, the search for signal molecules mediating stress tolerance is an important step toward better understanding how plants respond to pollutants. Phytohormones are active members of the signal cascade involved in the induction of plant stress response [2]. For example, abiotic stress results in both alerted levels of plant hormones and decreased plant growth [3]. The decreased cytokinin and gibberellic acid (GA_3) and increased abscisic acid contents are often observed responding in plants subjected to environmental

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stresses [4, 5]. Exogenous aplication of plant growth regulators could be an alternative strategy to ameliorate abiotic stress factors.

Gibberellins are a numerous group of plant hormones that in addition to auxins are one of the main groups of plant regulators [6, 7, 9]. They all differ in physiological activity and structure, and the first identified gibberellin was gibberellic acid (GA_3). Gibberellins are extensively involved in all phases of plant growth and development, from seed germination to senescence. They promote seed germination, stimulate stem elongation, leaf expansion, flowering, pollen, and seed development, delay ripening, and inhibit senescence [8-11]. Moreover, gibberellins are also involved in plant adaptation to abiotic stresses. For example, GA_3, the most active gibberellin, counteracts with saline soil conditions by improving membrane permeability and nutrient levels, which ultimately leads to better maize (*Zea mays*) seedling growth and establishment under toxic factors [3].

Green unicellular algae, with high metabolism rates such as *Chlorella vulgaris* (Chlorophyceae), play the basic role in the primary production and concentration of heavy metals, which are pollutants with multiple intake pathways to aquatic ecosystems. The danger of heavy metal pollution is due to its ability to circulate within aquatic and nearshore ecosystems for a prolonged length of time [12, 13]. Heavy metals can cause adverse effects on aquatic plant growth, cell division, respiration, photosynthesis and degeneration of the main cell organelles [14-17]. By accumulating heavy metals in their cells, algae promote further accumulation in the subsequent parts of the food chain, including in commercial aquatic organisms [18, 19]. In this respect, study of the effects of the most dangerous metals (Cd, Pb) on the growth of green algae species as a crucial component of natural phytocenoses seems important.

For this reason, the effect of exogenous GA_3 on the growth, bioaccumulation of heavy metals, and biochemical changes in *C. vulgaris* exposed to phytotoxic concentrations of Cd and Pb was examined. Obtained results may be essential for a better understanding of the plant hormone role in biochemical adaptation of green microalgae to stress conditions present in polluted water ecosystems.

Experimental Procedures

Plant Material and Growth Conditions

C. vulgaris culture was obtained from the collection of the Department of Plant Biochemistry and Toxicology at the University of Białystok, Poland. Microalgae were cultivated in stable conditions, where humidity amounted to 45(±5)%, and temperature amounted to 25(±1)ºC. Illumination was supplied during a 16-h photoperiod (8-h dark period) by a bank of fluorescent lights yielding a photon flux density of 50 μmol $m^{-2} \cdot s^{-1}$ of photosynthetically active radiation (PAR) at the surface of the tubes. PAR was measured with a phytophotometre FF-01 (SOMOPAN, Poland). Permanent synchronous growth was established according to the method of Pirson and Lorenzen [20]. The culture medium used was modified Knop's medium with the following components: 0.5 g KNO_3, 0.5 g $Ca(NO_3)_2 \cdot 4H_2O$, 0.2 g KH_2PO_4, 0.15 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeCl_3 \cdot 6H_2O$, 0.003 g H_3BO_3, 0.002 g $MnCl_2 \cdot 4H_2O$, 0.0003 g NH_4VO_3, 0.0002 g $ZnSO_4 \cdot 7H_2O$, and 0.0001 g $(NH_4)_6Mo_7O_{24} \cdot 7H_2O$ per 1 L of distilled water. The culture medium was sterilized by autoclaving at 125ºC for 25 min. In additon, glassware and bacteriological stoppers were sterilized in a thermal chamber at 105ºC for 4 hours. The pH of the medium had to be stable at 6.80(±0.1). Algae were cultured in Erlenmeyer flasks containing 250 mL of medium. The culture from which the inoculum was taken was in logarithmic growth phase.

In the experiment, the effect of Cd and Pb at the range of concentrations 10^{-7}-10^{-4} M with the stable concentration of GA_3 (10^{-5} M) was analyzed. The appropriate amounts of cadmium chloride and lead nitrate (Sigma-Aldrich Co., USA) were dissolved in distilled water and then added in correct concentrations to Erlenmeyer flasks with Knop's medium, algal suspension and GA_3 (10^{-5} M). Heavy metals content, cell number and proteins, monosaccharide and photosynthetic pigment levels in response to GA_3 and heavy metals were analyzed. The cell number and biochemical parameters were determined on days 1, 2, and 3 of cultivation. Tretments were conducted using four replicates.

Determination of Cadmium and Lead in *Chlorella vulgaris* Cells

To designate the concentration of heavy metals in algae, cultures of *C. vulgaris* were centrifuged at 3,500 rpm for 10 minutes. The supernatant was removed, and the suspension of *C. vulgaris* was mineralized. After this, samples were dissolved in 2 mL nitric acid and the concentration of cadmium and lead were analyzed by a Solaar M6 (Thermo Electron Comporation, UK) atomic absorption spectrometer with deuterium background correction system. The absorbances of Pb and Cd were measured in air-acetylene flame with 0.5 nm spectral bandpass at λ=217.0 nm and λ=228.8 nm, respectively.

Determination of Cell Number

The number of *C. vulgaris* cells was determined by direct counts of cells in the growth medium using a Bürker chamber.

Determination of Proteins

The concentration of proteins was determined spectrophotometrically by Lowry et al. [21], using Folin phenol reagent with a protein kit calibrated with bovine serum albumin as the standard. The absorbance of the extracts was measured with a Shimadzu UV-Vis 1201 spectrophotometer.

Table 1. The concentration of endogenic cadmium and lead in *Chlorella vulgaris* cells treated with heavy metals and GA_3 (fg/cell^{-1}).

Concentration of exogenous heavy metals and GA_3	Day of cultivation		
	1	2	3
0 (control)	0	0	0
10^{-5} M GA_3	0	0	0
10^{-4} M Cd+10^{-5} M GA_3	151 (±10.1)	354 (±12.5)	589 (±20.1)
10^{-5} M Cd+10^{-5} M GA_3	128 (±5.8)	295 (±7.8)	471 (±18.2)
10^{-6} M Cd+10^{-5} M GA_3	117 (±4.7)	235 (±4.3)	319 (±11.5)
10^{-7} M Cd+10^{-5} M GA_3	83 (±3.2)	116 (±5.1)	217 (±4.9)
10^{-4} M Pb+10^{-5} M GA_3	135 (±7.6)	278 (±9.5)	498 (±11.8)
10^{-5} M Pb+10^{-5} M GA_3	116 (±5.1)	209 (±8.8)	328 (±7.3)
10^{-6} M Pb+10^{-5} M GA_3	87 (±2.6)	115 (±4.3)	139 (±8.5)
10^{-7} M Pb+10^{-5} M GA_3	35 (±0.9)	51 (±1.9)	77 (±2.2)

Determination of Monosaccharide

The concentration of monosaccharide was determined spectrophotometrically using Somogyi and Nelson's [22] method with an arsenomolybdate reagent. Initially, 10 ml subsamples of the algal culture were collected by centrifuge at 3,500 rpm for 10 minutes. Then the monosaccharides were extracted in ethanol for 24 h. Absorbance was measured with a Shimadzu UV-Vis 1201 spectrophotometer.

Determination of Photosynthetic Pigments

The content of photosynthetic pigments (chlorophyll *a* and *b*, carotenoids) followed homogenization of fresh *C. vulgaris* in 99.9% methanol at 70ºC for 30 min [23]. The absorbance of the extract was measured with a Shimadzu UV-Vis 1201 spectrophotometer at 652.4 and 665.2 nm for chlorophylls *a* and *b,* and at 470.0 nm for carotenoids. The amounts of photosynthetic pigments present in the methanol extract were calculated according to the equations of Wellburn [23].

Replication and Statistical Analysis

Each treatment consisted of 4 replicates and each experiment was carried out on at least two different occasions. A minitab statistical package was used to carry out a one-way ANOVA. The Student's t-test was used to estimate the difference between means at a 5% level of significance.

Results

Previous research [16, 24] indicated that *C. vulgaris* cells bioaccumulate toxic metals from polluted water in a dose- and exposure-dependent manner. The biosorption of heavy metals by green algae is accompanied by an induction of a variety of cellular changes, such as degradation of metabolites and growth inhibition. Algal adaptation to the heavy metals may depend on the effect of the exogenous phytohormones that directly contribute to metal tolerance capacity of the plant. Therefore, the effect of 10^{-5} M GA_3 on Cd and Pb accumulation, cell number, and the contents of photosynthetic pigments, proteins, and monosaccharides in the culture of *C. vulgaris* exposed to phytotoxic heavy metals (10^{-7}-10^{-4} M) were examined. Preliminary experiments indicated that GA_3 applied at a concentration of 10^{-5} M was characterized by the most favorable stimulation on the cell number, protein, chlorophyll, carotenoid and monosaccharide level in microalgae (data not shown).

The results showed that Cd and Pb level in *C. vulgaris* culture depends on the length of cultivation and concentration of applied heavy metals (Table 1). Green algae exposed to both 10^{-4} M Pb and Cd plus 10^{-5} M GA_3 contained the highest amounts of these metals (589 fg^{-1} Cd cell and 498 fg^{-1} Pb cell) after 3 days of culture. This increase was six-fold in the case of Cd and almost triple for Pb. Therefore, obtained data indicated that Cd, in the observed concentration ranges of 10^{-7}-10^{-4} M, was accumulated more effectively in comparison to Pb in cells growing in the presence of gibberellic acid.

During the entire time of the experiment, GA_3 stimulated the growth of *C. vulgaris* exposed to Pb and Cd at a range of concentrations 10^{-7}-10^{-5} M in the medium (Fig. 1). The increase by 41% and 39% in cell number of microalgae treated with 10^{-7} M Pb and 10^{-5} M GA_3, as well as 10^{-7} M Cd and 10^{-5} M GA_3, respectively, was noted on day 3 of cultivation. Moreover, the slight inhibitory influence of 10^{-5} GA_3 on the cell number was observed in response to both 10^{-4} M Cd and Pb. However, in the case of 10^{-4} M Cd, algal growth inhibition was not statistically significant because the cell number was similar to the control. Conversly, 10^{-5} M GA_3 induced a 30% increase in cell numbers on day 3 of cultivation.

The exogenous application of 10^{-5} M GA_3 caused a 51% increase in protein content in *C. vulgaris* on day 3 of cultivation. Treatment with GA_3 (10^{-5} M) and heavy metals at lower concentration ranges of 10^{-7}-10^{-5} M stimulated protein accumulation in the algal culture. The increase in protein level by 40% (Cd) and 67% (Pb) was observed at the lowest concentration (10^{-7} M) of heavy metals and 10^{-5} M GA_3 on day 1 (Cd) and day 3 (Pb) of cultivation (Fig. 2). By contrast, the inhibitory (but not statistically significant) influence of Cd and Pb on the protein amount in microalgae at the presence of toxic concentration (10^{-4} M) of heavy metals was noted. Examined metals at the highest dose and 10^{-5} M GA_3 caused a slight reduction in the content of protein by 8% (Cd) and by 5% (Pb) on day 3 of cultivation.

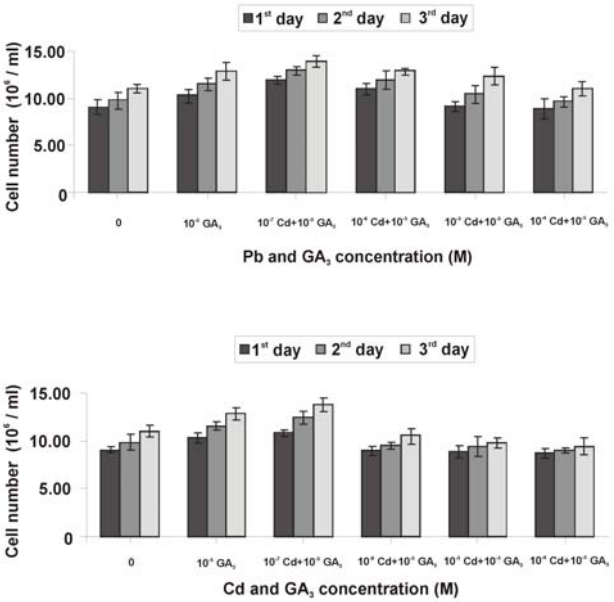

Fig. 1. The effect of gibberellic acid GA_3 (10^{-5} M) on cell number in *Chlorella vulgaris* treated with cadmium and lead (10^{-7}-10^{-4} M). Data are the means of four independent experiments ± SE.

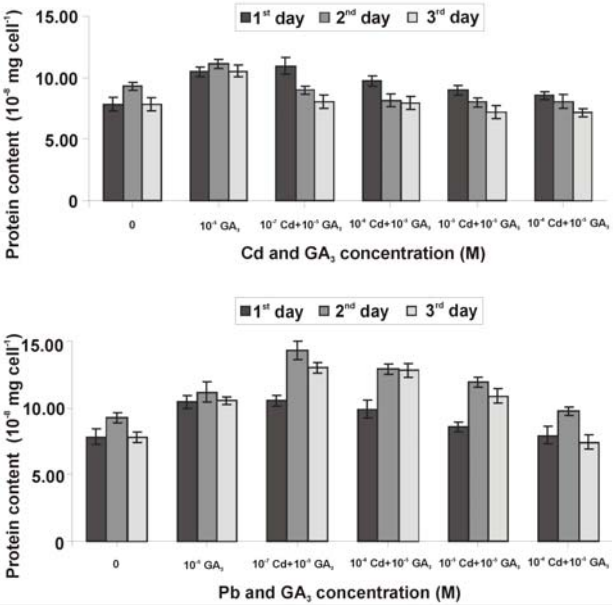

Fig. 2. The effect of the gibberellic acid GA_3 (10^{-5} M) on proteins in *Chlorella vulgaris* treated with cadmium and lead (10^{-7}-10^{-4} M). Data are the means of four independent experiments ± SE.

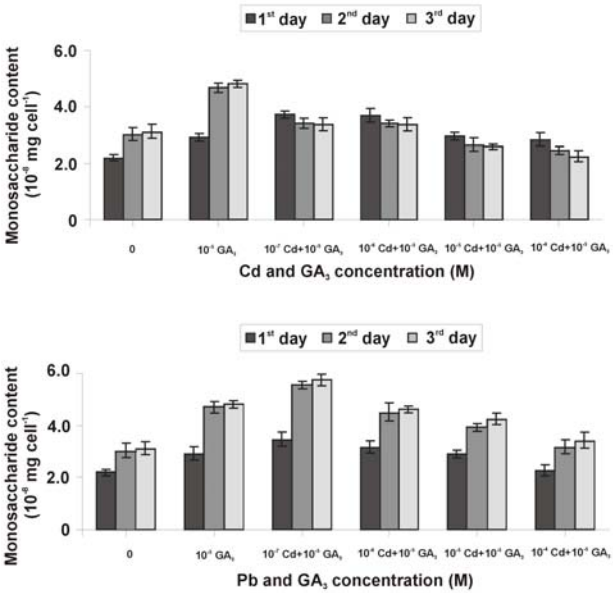

Fig. 3. The effect of the gibberellic acid GA_3 (10^{-5} M) on monosaccharide in *Chlorella vulgaris* treated with cadmium and lead (10^{-7}-10^{-4} M). Data are the means of four independent experiments ± SE.

The experiment showed that monosaccharide content was stimulated by 57% in response to 10^{-5} M GA_3 on day 2 of cultivation (Fig. 3). Treatment with 10^{-5} M GA_3 reduced the toxic effect of heavy metals on this biochemical parameter, especially at a range of concentrations 10^{-7}-10^{-6} M of Cd and Pb. The highest stimulation of monosaccharide accumulation, by 70%, was observed under the influence of 10^{-5} M GA_3 and 10^{-7} M Cd on day 1 of incubation. The highest increase (57-85%) in the amounts of sugars was noted in the presence of 10^{-7} M Pb and 10^{-5} M GA_3. 10^{-5} M GA_3 and Pb added at the highest concentration (10^{-4} M) caused a 2-10% increase in monosaccharide levels. The inhibitory action was observed in the case of 10^{-4} M Cd and 10^{-5} M GA_3, which reduced monosaccharide amount by 18-27% in relation to the control.

The experiment showed that GA_3 stimulated by 64% (day 3) and 42% (day 1) the accumulation of chlorophyll *a* and *b* (Fig. 4). Moreover, the application of 10^{-7} M Cd with GA_3 (10^{-5} M) to *C. vulgaris* culture resulted in a 58% increase in chlorophyll *a* levels on day 2 and in chlorophyll *b* content by 50% on day 1 of the experiment in relation to the control. Similarly, the addition of 10^{-5} M GA_3 and Pb at 10^{-7} M resulted in a 10% increase in the content of chlorophyll *a* and chlorophyll *b* by 35% on day 3. GA_3 at 10^{-5} M did not reduce the toxic effect in the case of lead because of a decrease in chlorophyll levels at 10^{-6} M. Treatment with 10^{-5} M GA_3 and Pb at 10^{-4} M induced a decrease in chlorophyll *a* (by 27% on day 1) and chlorophyll *b* (by 20% on day 2) accumulation.

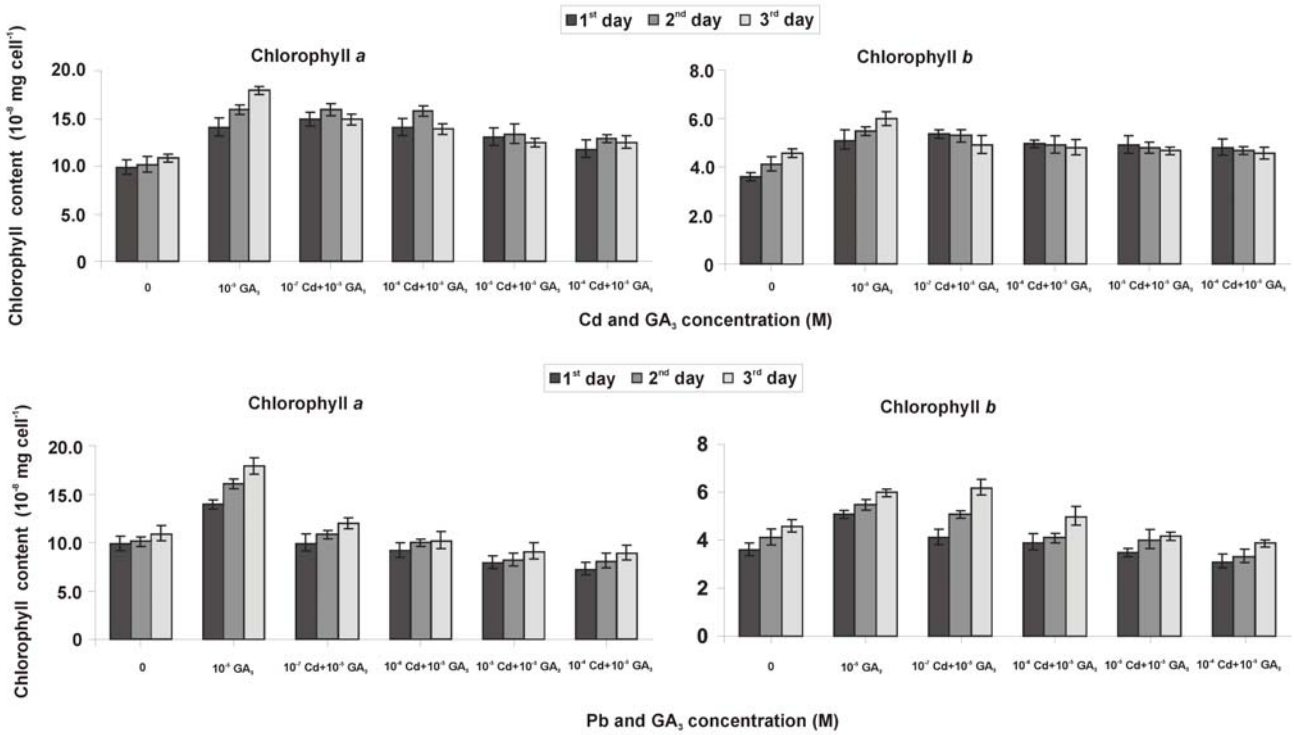

Fig. 4. The effect of gibberellic acid GA_3 (10^{-5} M) on chlorophyll *a* and *b* in *Chlorella vulgaris*, treated with cadmium and lead (10^{-7}-10^{-4} M). Data are the means of four independent experiments ± SE.

The amount of carotenoids in algal cells under the influence of 10^{-5} M GA_3 depends upon Cd and Pb dose as well as cultivation time (Fig. 5). The higher the concentration of heavy metal, the less the amount of carotenoids in *C. vulgaris*. A different relationship was observed in the case cultivation time, in which longer cultivation time directly correlated with an increase in the amount of carotenoids. Therefore, the increase in carotenoid levels by 36% (day 1) and 20% (day 1) was observed in response to 10^{-7} M Cd and 10^{-5} M GA_3, as well as 10^{-7} M Pb and 10^{-5} M GA_3, respectively. However, Pb at concentrations of 10^{-5}-10^{-4} M plus 10^{-5} M GA_3 reduced the amount of carotenoids in *C. vulgaris*. By contrast, the amount of carotenoids in *C. vulgaris* was stimulated by 29% (day 3) in response to 10^{-5} M GA_3.

Discussion

Phytohormones play an important role in plant metabolism. However, most of the currently conducted research relates to higher plants. There are not many articles about the biochemical role of gibberellic acid (GA_3) in algae, especially in *Chlorella vulgaris*. Therefore, in this case research was conducted on the influence of GA_3 on the growth, metal bioaccumulation, and biochemical composition of *C. vulgaris*. *C. vulgaris* (Chlorophyceae) is used as a model system because it can be cultivated in simple media and is characterized by rapid cell division. Moreover, variations in signalling molecules and biochemical responses can be perceived in the same cell.

Gibberellins, which are one of the plant regulators, have a huge influence on the basic developmental plant processes [7]. This research was conduced to determine if the fundamental parameters such as cell number, proteins, monosaccharide, chlorophyll *a* and *b,* and carotenoids will be stimulated by GA_3, despite exposure to heavy metals.

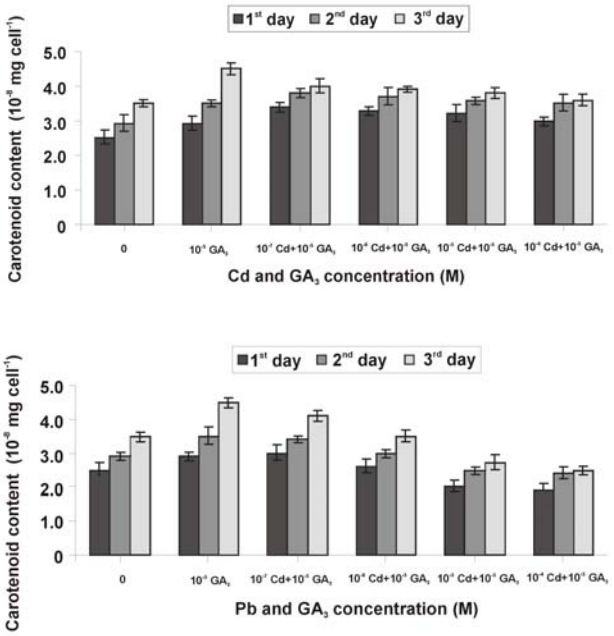

Fig. 5. The effect of the gibberellic acid GA_3 (10^{-5} M) on carotenoid in *Chlorella vulgaris*, treated with cadmium and lead (10^{-7}-10^{-4} M). Data are the means of four independent experiments ± SE.

The experiment indicated that the concentration of heavy metals and cultivaton period were two factors that had a huge impact on GA_3 activity. GA_3 at a concentration 10^{-5} M and with exposure to both heavy metals at the lowest concentration (10^{-7} M) caused the most significant increase in all the measured parameters. Cadmium and lead at 10^{-4} M ihibited activity on the analyzed metabolites. Therefore, gibberellic acid does not withstand the strong toxic concentration of heavy metals and it had a negative effect on *Chlorella vulgaris* growth. The inhibitory activity of the analyzed metabolites became weaker with decreasing concentrations of heavy metals.

Water plants have a huge ability to accumulate and bioconcentrate exogenic heavy metals. Lethal concentrations of lead for *C. vulgaris* amounted to $5 \cdot 10^{-3}$ M for non-organic lead and $7.5 \cdot 10^{-6}$ M for organic lead [25]. The results presented here indicate that both heavy metals, at the concentrations in the range of 10^{-7}-10^{-4} M, were collected and accumulated in *C. vulgaris* cells very quickly, even on the first day of the experiment. The highest level of endogenic cadmium and lead was observed in cells that were treated with 10^{-4} M Cd and Pb. The lethal effect of these heavy metals were not bridged even with the addition of GA_3. The content of heavy metals in cells of *C. vulgaris* gradually increased with the passage of experimental days. The results show that the bioaccumulation of cadmium was greater in comparison with lead. Experiments with *Scenedesmus quadricauda* proved that the algae biosorbed and bioconcentrated heavy metals from the aquatic environment in the following order: $Cd^{2+} > Hg^{2+} > Cr^{6+} > Pb^{2+} > As^{5+}$ [26].

The results presented here demonstrate that GA_3 had a stimulating influence on the cell number, despite treating *C. vulgaris* with various concentrations of heavy metals. But this stimulating activity diminished along with the growing concentrations of cadmium and lead, inhibiting the growth and development of algae. So the most inhibiting effect was observed in the presence of 10^{-4} M of both Cd and Pb. The results proved that GA_3 withstood the toxic concentrations of cadmium and lead based upon the cellular division (cell number) of *C. vulgaris*. The experiments with other species of algae (*Stigeoclonium tenue*) [27] demonstrated that those organisms adapted to ecosystems with high concentrations of heavy metals accumulated heavy metals intracellularly. Besides, they becaame more resistant. The research by Jianga and Liu [28] proved that lead was very destructive. Lead at concentrations of 10^{-2} and 10^{-3} M inhibited cell division in *Zea mays* within 24 h. The results presented here indicate that *C. vulgaris* characterized a huge sensitivity to heavy metals action, but gibberellic acid had reduced this toxic influence of cadmium and lead. Therefore, cell division and cell numbers increased during the experiment.

The results demonstrate that GA_3 addition to *C. vulgaris* culture treated with heavy metals stimulated the protein content. It is known that there is a group of proteins that bind ions of heavy metals through the thiol group of cysteine residues. These proteins can protect plant cells against the toxic influence of heavy metals [16, 29]. So it can be speculated that GA_3 activated defense reactions and catalyzed synthesis and accumulation of phytochelatins that participated in heavy metal detoxification [30]. The results indicated that the concentration of proteins maximized on the first day of cultivation (in the case of cadmium) and in the second day (in the case of lead). The highest increase of protein content was at the concentration of 10^{-7} M Cd and Pb, and the greatest decrease was observed at the concentration of 10^{-4} M Cd and Pb.

From previous research it appears that monosaccharides play an energetic and building function in plants. The research by Grill et al. [30] proved that heavy metals at the concentration of 10^{-4} M caused the strongest cohabitation of monosaccharide synthesis in the cells of algae. The results presented here demonstrate the same relation. Cadmium was the only heavy metal that caused a drop in the level of monosaccharides. It was observed at the range of concentration 10^{-5}-10^{-4} M Cd. Conversly, the amount of monosaccharides did not decrease after lead treatment in comparison to the control culture.

Previous research found that heavy metals have a negative effect on chlorophyll *a* and *b* synthesis. The results presented here demonstrated that GA_3 stimulated the amount of the photosynthetic pigments. The highest increase of chlorophyll content was observed on day 2 (chlorophyll *a*) and on day 1 (chlorophyll *b*) of cultivation after cadmium treatment. Moreover, lead caused the greatest increase of chlorophyll *a* and *b* on day 3 of cultivation. The research by Stiborova and others [31] with barley leaves proved that chlorophyll *b* was more inhibited in comparison with chlorophyll *a*. This is in agreement with our research. The concentration of total carotenoids was at a high level over the entire time of the experiment. Carotenoids are important pigments because they protect the protein of photosystem II and chlorophylls from degradation. They also protect the photosynthetic apparatus against photooxidation and against the negative effect of ultraviolet light [32]. Along with the cultivation time of the experiment, cells of *C. vulgaris* were becoming older and were losing their green color. Probably, chlorophyll converts into pheophytin because pheophytin *a* and *b* are one of the direct products of the biochemical transformation of chlorophylls, which in extended stages can be fully degraded. Rai and the others [33] presented the idea that synthesis of chlorophylls is inhibited by lead. Research on *Nostoc muscorum* showed that the sensitivity of photosynthetic pigments to heavy metals are represented in the following: chlorophyll>phycocyanin>carotenoids [33]. Our research results demonstrated that chlorophylls are degraded in the first stage and then carotenoids.

Conclusions

Stress can cause various responses in plants, which on the one hand can cause irreversible disturbances in structure and cell functions, but on the other hand can increase resistance to many of these stresses. Results of this study

demonstrate that GA_3 will confer tolerance in microalgae (*Chlorella vulgaris*) to exposure to low concentrations of heavy metals. Moreover, plants from Chlorophyta can be a useful group of organisms for accumulating and bioconcentrating heavy metals in phytoremediation techniques.

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