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Protective role of 20-hydroxyecdysone against lead stress in *Chlorella vulgaris* cultures

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Abstract

Treatment of cultured *C. vulgaris* cells with 10^{-6}–10^{-4} M lead decreased their growth and chemical composition during the first 48 h of cultivation. However, at concentrations above 10^{-4} M, lead is cytotoxic to *Chlorella vulgaris* cells, resulting in cellular fragmentation and lysis. In contrast, at concentrations below 10^{-6} M lead had no influence on the growth and metabolism of *C. vulgaris* cells. 20-Hydroxyecdysone (20E) (10^{-10}–10^{-8} M) increased growth and chemical composition of *C. vulgaris* cells over a concentration range. Levels per cell of chlorophylls, protein, sugars are all increased by 20E treatment, when compared to non-treated control cells. However, the cultures treated with 20E and lead show a lower stimulation than the cultures treated with 20E alone. The effects of 20E mixed with lead on the growth and the level of cellular lead, chlorophyll, sugar and protein in *C. vulgaris* are also reported. The decreased growth and composition of *C. vulgaris* cells treated with lead was restored by the 20E. Application of 20E to *C. vulgaris* cultures reduced the impact of lead stress on growth, prevented chlorophyll, sugar and protein loss and increased phytochelatins synthesis. Furthermore, 20E did not restore toxic effect of lead on *C. vulgaris* cells. The combined treatment with lead and 20E appeared to have a stimulatory effect on the above parameters during the 48 h of cultivation, as compared to the control. 20E reduced the toxicity of lead and the growth recovered to the level of cells treated with 20E alone. Concentration-dependent stimulation was observed with increasing concentration of 20E and decreasing concentration of lead.

Keywords: *Chlorella vulgaris*; Trebouxiophyceae; Alga; Growth; 20-Hydroxyecdysone; Lead stress; Phytochelatins

1. Introduction

Environmental pollution caused by intensive activity of man is a great threat to the functioning of ecosystems. Pollution of aquatic environment with heavy metals presents potential health risks. Metals are non-degradable and can accumulate and concentrate as they move up the food chain. The toxic effect of heavy metals on plant growth and development is commonly known. Inhibition of growth limited photosynthesis and respiration. Inhibited biosynthesis of chlorophyll and carotenoids, reduced phosphorylation are most frequently observed symptoms of metal toxicity. In this connection, algae have been commonly used as test organisms because of their ecological importance as primary producers of most aquatic food chains. Lead in environment can cause serious problems for plants and animals. It has become a major environmental contaminant due to rapid industrialization and urbanization. Lead is not included in essential elements for plants, but they absorb this metal. In response to increasing pollution with heavy metals plants have developed mechanisms of stress avoidance or its tolerance, one of which employs cysteine-rich compounds, phytochelatins (PC) (Vymazal, 1990; Wilde and Benemann, 1993; Rauser, 1995; Vlchez et al., 1997; Cobbett, 2000; Cobbett and Goldsbrough, 2002). We present the inhibitory effect of lead (10^{-6}–10^{-4} M) on the growth (expressed as a number of cells), content of PC, chlorophyll, sugar and protein in *Chlorella vulgaris* cells, which was a secondary aim of this study.

The primary aim of this study was to examine the effect of ecdysteroid, 20-hydroxyecdysone (20E) at concentrations 10^{-10}–10^{-8} M on the content of cellular

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lead, chlorophyll, sugar and protein in lead-treated *C. vulgaris* cultures. Also the effects of 20E mixed with lead on the levels of PC in *C. vulgaris* cells have been studied. To our knowledge this is the first evidence that the inhibition in lead-treated *C. vulgaris* cells can be reversed by application of 20E. 20E is a member of ecdysteroids, which are the steroid hormones of arthropods and possibly of other invertebrates too. Furthermore, ecdysteroids are one of the various classes of steroidal compounds of plants. They might have a hormonal role in the plant. However, the evidence for this is not convincing yet (Lafont, 1998; Dinan, 2001). In insect larvae, ecdysteroids are excreted *via* the gut or Malpighian tubules. It can be found in the faeces as unchanged hormone and a mixture of metabolites. The excretion of ecdysteroids by aquatic-living insects into the water is possible (Isaac and Slinger, 1989). Also, there are other possible sources of ecdysteroids in aquatic environment, for example freshwater plants, crustacea or fungi. The effect of exogenous ecdysone on growth and the level of cellular components in *C. vulgaris* has been reported (Bajguz and Koronka, 2001). Furthermore, the application of three ecdysteroids, 20E, 2-deoxy-20E and 20E 22-acetate, on growth and development of *C. vulgaris* cells will be presented elsewhere (Bajguz and Dinan, unpubl.).

2. Results

This paper presents a study concerning the influence of 20-hydroxyecdysone (20E) and/or lead upon the growth and chemical composition of the green alga *C. vulgaris*. With regard to previous papers (Bajguz, 2000a,b, 2002a; Bajguz and Koronka, 2001) the concentrations 10^{-10}–10^{-8} M 20E and 10^{-6}–10^{-4} M lead were selected. Data were determined for each of the parameters (number of cells, chlorophyll, sugar and protein content) on hours 12, 24, 36 and 48 after the initiation of 20E and/or lead treatment. On the other hand, the content of cellular lead and PC was determinated on hours 1, 3, 6, 12, 24, 36 and 48 because these parameters presented also the changes at the first 12th h of cultivation. The effects of 20E in combination with lead on the growth and accumulation of this heavy metal by algal cells, and the cellular content of PC, chlorophyll, sugar, protein were determined by adding 10^{-10}–10^{-8} M 20E with three concentrations of metal (10^{-6}–10^{-4} M).

Lead, at a concentration of 10^{-3} M, alone or mixed with 20E, showed a lethal effect on the *C. vulgaris* cells after 5 min. Furthermore, at concentrations below 10^{-6} M lead had no influence on the growth and chemical composition of *C. vulgaris* cells (data not shown).

2.1. Growth of C. vulgaris cells

Addition of 10^{-6}–10^{-4} M lead to *C. vulgaris* cells reduced their growth during the first 48 h of cultivation (Fig. 1). However, treatment with lead at concentrations 10^{-6}–10^{-4} M results in growth levels very similar to those of control cell cultures (non-lead-treated) at the 12th h of cultivation. Furthermore, 10^{-6} M lead had no inhibitory effect on growth at the 48th h of cultivation. Lead mostly reduced *C. vulgaris* growth at 10^{-5}–10^{-4} M, during the 48 h of cultivation, at a concentration of 10^{-4} M lead showing the greatest growth-inhibitory effect in *C. vulgaris* cultures.

The most stimulating influence upon the number of cells of *C. vulgaris* was shown by 20E at a concentration of 10^{-8} M between the 12th and 48th hour of cultivation, increasing cell density by 129–148%, relative to the control (100%). In turn, the stimulation by 20E, at a concentration of 10^{-10} M between the 12th and 48th hour, was amounting to 112–126% of the control value. The phase of rapid growth of *C. vulgaris* was characteristic for the cells treated with 20E or not (control).

From 12th to the 48th hour of cultivation, application of a mixture of 10^{-8} M 20E and lead to *C. vulgaris* culture resulted in an increase in the number of cells, as compared to untreated cells. On the other hand, treatment with a higher concentration of lead (10^{-4} M) and 10^{-10}–10^{-9} M 20E resulted in weaker suppression of *C. vulgaris* growth at the 12th hour of cultivation. After 36 h of cultivation, a treatment of *C. vulgaris* cells with both 10^{-6}–10^{-4} M lead and 10^{-10}–10^{-8} M 20E showed a stimulatory effect on the growth in respect to the control. The combination of 10^{-6} M lead and 10^{-8} M 20E appeared to have the highest stimulatory effect on the number of cells. However, the cultures treated with 20E and 10^{-5}–10^{-4} M lead show a lower increase of the number of cells than the cultures treated with 20E alone. Furthermore, application of a mixture of 20E and 10^{-6} M lead to a *C. vulgaris* culture resulted in growth levels very similar to that of cell culture treated with 20E alone.

2.2. Accumulation of lead in C. vulgaris cells

The content of lead in *C. vulgaris* cells was correlated to the concentration of metal ion in the cultures (Fig. 2). The interaction of 20E and lead showed a weaker accumulation by the algal cell than the culture treated with the metal alone. Cultures of *C. vulgaris* treated with 20E (in the range of concentrations 10^{-10}–10^{-8} M) and lead (10^{-6}–10^{-4} M) demonstrate the reduction of this heavy metal accumulation. The combination of 10^{-4} M lead and 10^{-8} M 20E had the highest reduction of lead accumulation. Ecdysteroid, in the range of concentration 10^{-10}–10^{-8} M, decreased the lead content in *C. vulgaris* cells, reaching the maximum at the 12th hour of

cultivation. The accumulation of lead was intensively decreased at a concentration of 10^{-8} M 20E at the 12th hour of cultivation. The lowest activity in reduction of lead content was demonstrated at a concentration of 10^{-10} M 20E. In dose decreasing-dependent fashion, the inhibitory effect of 20E was observed between the 12th and 48th hour of cultivation. However, the differences between lead content of cells treated simultaneously with 20E are rather small. Probably, different concentrations of 20E do not play an important role in the uptake of lead, its inhibition and synthesis of PC as the answer of *C. vulgaris* to heavy metal exposition.

2.3. Phytochelatins content in C. vulgaris cells

Cell treated with lead alone showed a weaker increase of total PC content (Fig. 3) between the 1st and the 12th hour of cultivation (the ratio of PC:Pb is $<1{:}1$ and 2:1, respectively; Fig. 4) than the culture treated with 20E and lead. The stimulating effect of lead alone on PC content was observed from 12th to 48th hour, peaked at the 36th hour (the ratio of PC:Pb is $>20{:}1$; Fig. 4). On the other hand, 10^{-6} M lead had no effect on PC synthesis at the 48th hour of cultivation. The highest increase of PC content in lead-treated *C. vulgaris* cells was exerted at a concentration of 10^{-4} M and the weakest at 10^{-6} M (the ratio of PC:Pb is 10–30:1; Fig. 4).

20E together with lead increased the growth, which was correlated with an increase of total PC content (not shown). The maximal PC value is increased with the highest lead concentrations. An increase in lead concentration caused an increase in PC content in *C. vulgaris* cells, especially with interaction of 20E and at the first 12 h of cultivation. The most stimulating effect on PC content was shown by 10^{-8} M 20E with 10^{-4} M lead.

2.4. Chlorophyll content in C. vulgaris cells

Application of lead, at a range of concentrations from 10^{-6} to 10^{-4} M, to cultured *C. vulgaris* cells caused reduction of chlorophyll content that was apparent at the 48 h of cultivation (Fig. 5). In contrast, at a concentration of 10^{-6} M, lead had a slight inhibitory effect on the chlorophyll content in *C. vulgaris* cells as compared to the control culture. It had the greatest inhibitory effect at a concentration of 10^{-4} M.

20E in the range of concentrations 10^{-10}–10^{-8} M enhances the content of chlorophyll in *C. vulgaris* cells. The maximum stimulation of chlorophyll content occurs at a concentration of 10^{-8} M 20E between the 12th and 48th hour of cultivation.

The reduction of chlorophyll content in *C. vulgaris* cells treated with lead was prevented by the co-application of 10^{-10}–10^{-8} M 20E. The combination of 20E and lead showed a stimulating influence upon the content of chlorophyll in the cells of *C. vulgaris*, without regard to their concentrations or the period of the cultivation.

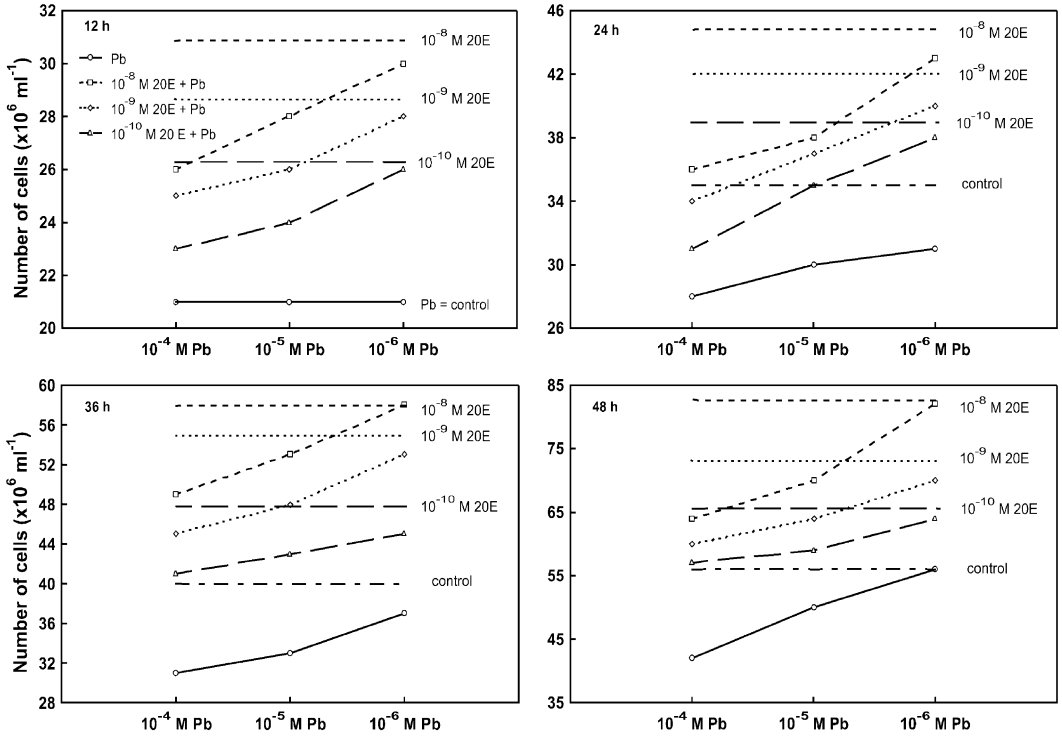

Fig. 1. Effect of lead in the absence or presence of 20E on the growth of *C. vulgaris* expressed as a number of cells. Data are means±SE of results from 15 replicate samples. Error bars were smaller than the symbols for most points. Before treatment of *C. vulgaris* cultures, the number of cells was established at 21×10^6 cells ml^{-1}.

Cultures of the algae *C. vulgaris* treated with 10^{-10} M 20E and 10^{-4} M lead are characterized by slight increase of the chlorophyll content. 20E, at a concentration of 10^{-8} M, had the greatest effect on the content of chlorophyll in lead-treated *C. vulgaris* cells at the 48th hour of cultivation. The cultures treated with 20E and 10^{-5}–10^{-4} M lead show a lower increase on the content of chlorophyll than the cultures treated with 20E alone. On the other hand, application of a mixture of 20E and 10^{-6} M lead to a *C. vulgaris* culture resulted in chlorophyll levels very similar to that of cell culture treated with 20E alone.

2.5. Sugar content in C. vulgaris cells

Application of 10^{-6}–10^{-4} M lead to *C. vulgaris* cells decreased the content of sugar during the first 48 h of cultivation (Fig. 6). The most significant reduction of sugar content in lead-treated *C. vulgaris* was observed at 10^{-5}–10^{-4} M, during the 48 h of cultivation, with a concentration of 10^{-4} M lead showing the greatest inhibitory effect. Furthermore, 10^{-6} M lead had no inhibitory effect on sugar content at the 48th hour of cultivation.

The most stimulating influence upon the content of sugar in *C. vulgaris* cells was shown by 20E at a concentration 10^{-8} M at the first 48th hours of cultivation, within the limits 174–248% with respect to the control. The data provided indicate that under the influence of 20E, at the range of concentrations 10^{-10}–10^{-8} M a significant increase in the sugar content occurs.

The inhibitory effect of lead on the content of sugar in *C. vulgaris* cells was suppressed by the co-application of 10^{-10}–10^{-8} M 20E. The combination of 10^{-6} M lead and 10^{-8} M 20E appeared to have the highest stimulatory effect on the sugar content after 48 h of cultivation, as compared to the control. However, this mixture of compounds showed a stimulatory effect, which was

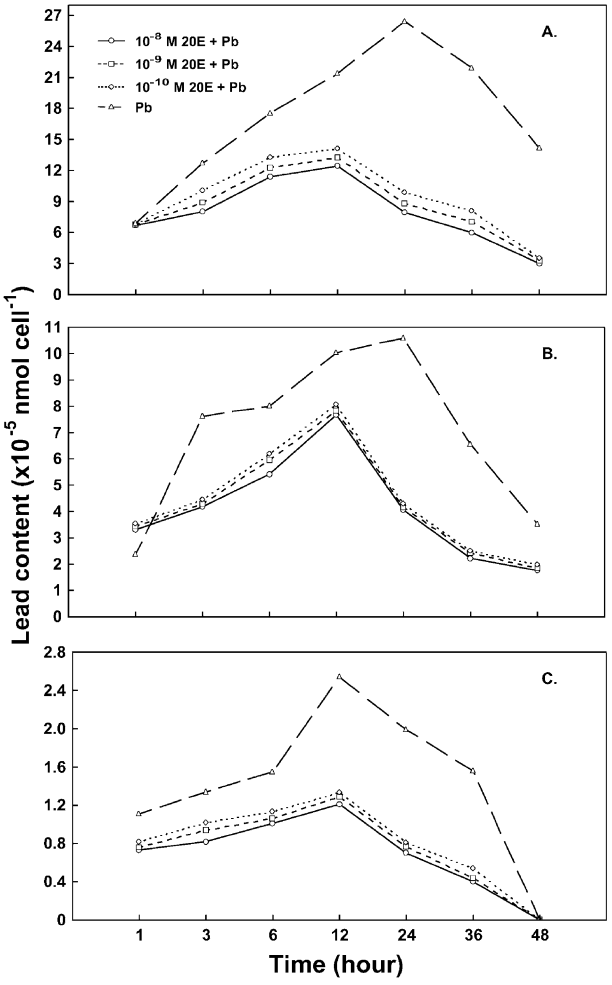

Fig. 2. Effect of lead (A, 10^{-4} M; B, 10^{-5} M; C, 10^{-6} M) in the absence or presence of 20E on the lead content in *C. vulgaris* cells. Data are means±SE of results from 15 replicate samples. Error bars were smaller than the symbols for most points. Before treatment of *C. vulgaris* cultures, the content of lead was established at 0. Control cultures during the 48 h of cultivation did not contain lead in cells.

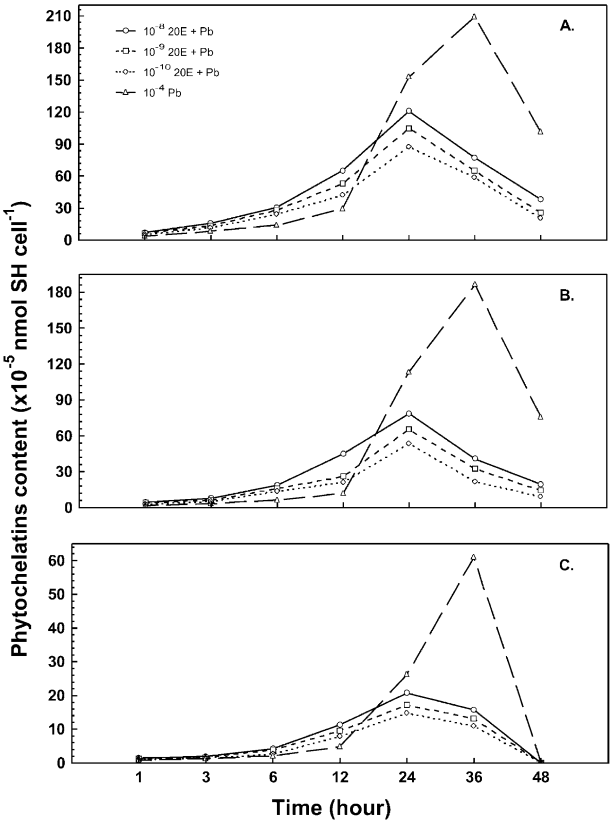

Fig. 3. Effect of lead (A, 10^{-4} M; B, 10^{-5} M; C, 10^{-6} M) in the absence or presence of 20E on the phytochelatins content in *C. vulgaris* cells. Data are means±SE of results from 15 replicate samples. Error bars were smaller than the symbols for most points. Before treatment of *C. vulgaris* cultures, the phytochelatins content was established at 0. Control cultures during the 48 h of cultivation did not contain phytochelatins in cells.

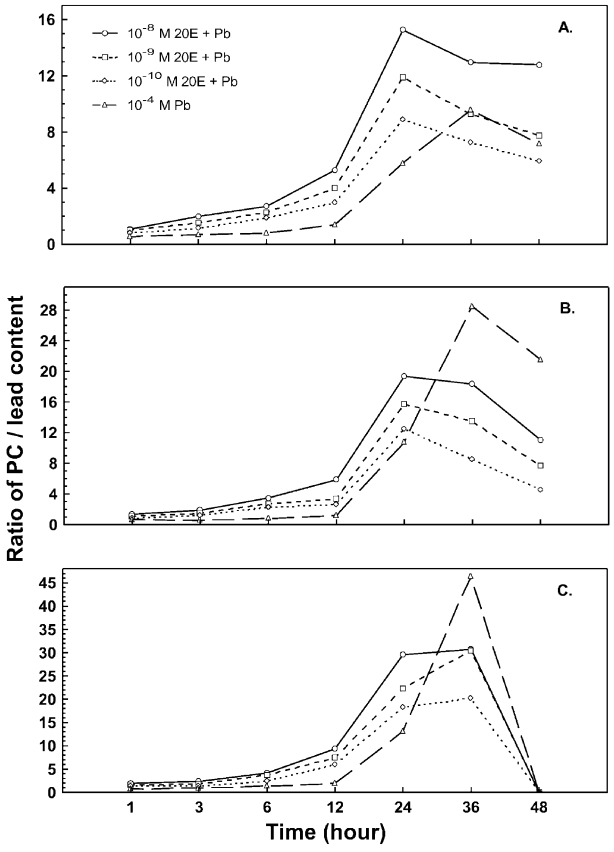

Fig. 4. Molar ratio of phytochelatins to lead content in the absence or presence of 20E in lead-treated (A, 10^{-4} M; B, 10^{-5} M; C, 10^{-6} M) *C. vulgaris* cells.

similar to that observed in cells treated with 20E alone. A decrease in lead concentration caused an increase in sugar content in *C. vulgaris* cells, especially with interaction of 20E.

2.6. Protein content in C. vulgaris cells

Lead displayed the greatest inhibitory activity at a concentration of 10^{-4} M between the 12th and 48th hour of cultivation (Fig. 7). The lowest activity in decreasing protein content was demonstrated at a concentration of 10^{-6} M lead. The inhibitory effect of lead on the protein content in *C. vulgaris* cells was suppressed by the co-application of 10^{-10}–10^{-8} M 20E. Cultures of the algae *C. vulgaris* treated with 10^{-10} M 20E and 10^{-4} M lead are characterized by slight increase of the protein content. 20E, at a concentration of 10^{-8} M, had the greatest stimulatory effect on the content of protein in lead-treated *C. vulgaris* cells. 20E in the range of concentration 10^{-10}–10^{-8} M increased the protein content of *C. vulgaris* cells, reaching the maximum at a concentration of 10^{-8} M. In *C. vulgaris* protein content is increased at the 48th hour of cultivation. In dose decreasing-dependent fashion, the stimulatory effect of 20E is observed in the range 10^{-10}–10^{-9} M. Furthermore, the potency of 20E and lead in increasing the content of protein with respect to *C. vulgaris* cells treated with 20E occurred in the same order as for the other parameters.

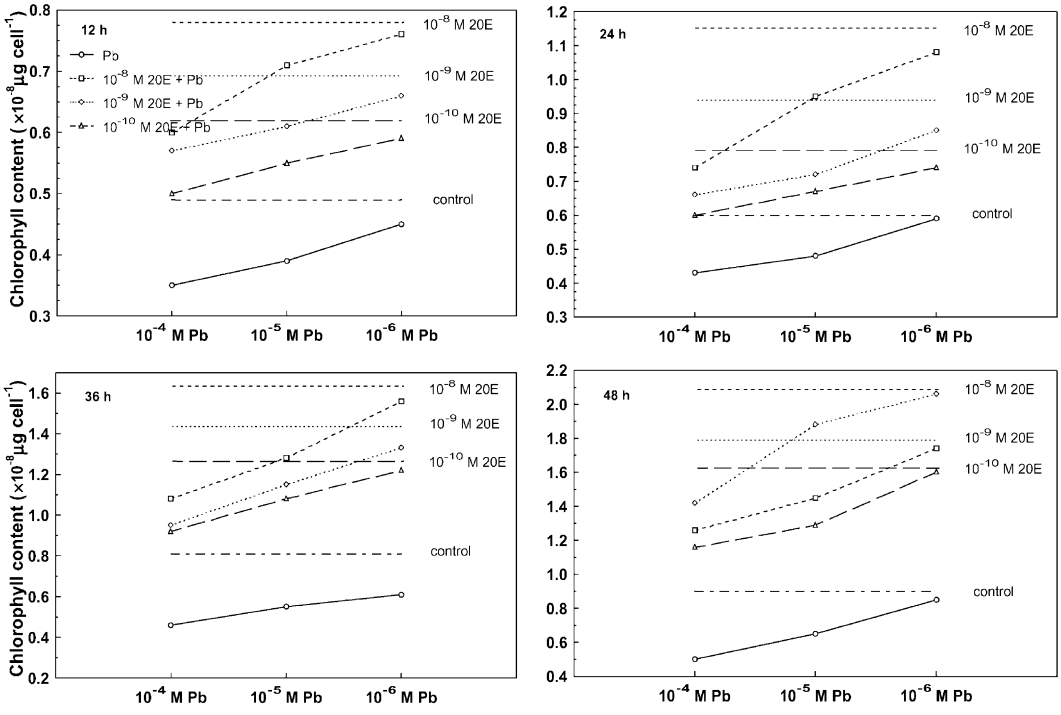

Fig. 5. Effect of lead in the absence or presence of 20E on the chlorophyll content in *C. vulgaris* cells. Data are means±SE of results from 15 replicate samples. Error bars were smaller than the symbols for most points. Before treatment of *C. vulgaris* cultures, the chlorophyll content was established at 0.35×10^{-8} µg cell^{-1}.

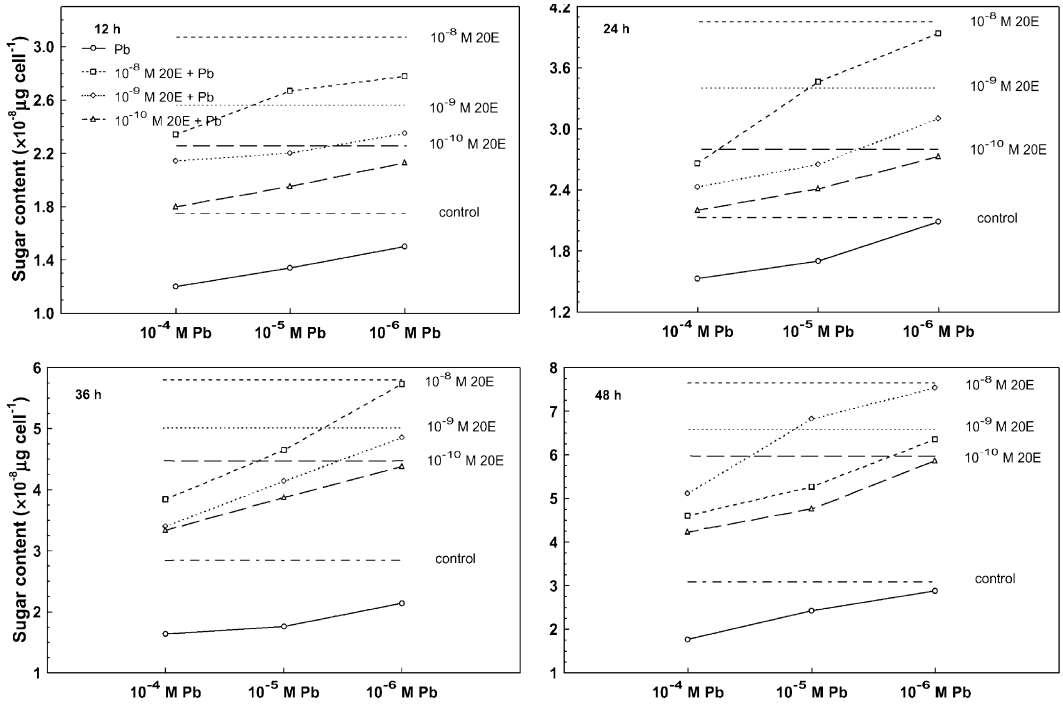

Fig. 6. Effect of lead in the absence or presence of 20E on the sugar content in *C. vulgaris* cells. Data are means±SE of results from 15 replicate samples. Error bars were smaller than the symbols for most points. Before treatment of *C. vulgaris* cultures, the sugar content was established at 1.20×10^{-8} µg cell^{-1}.

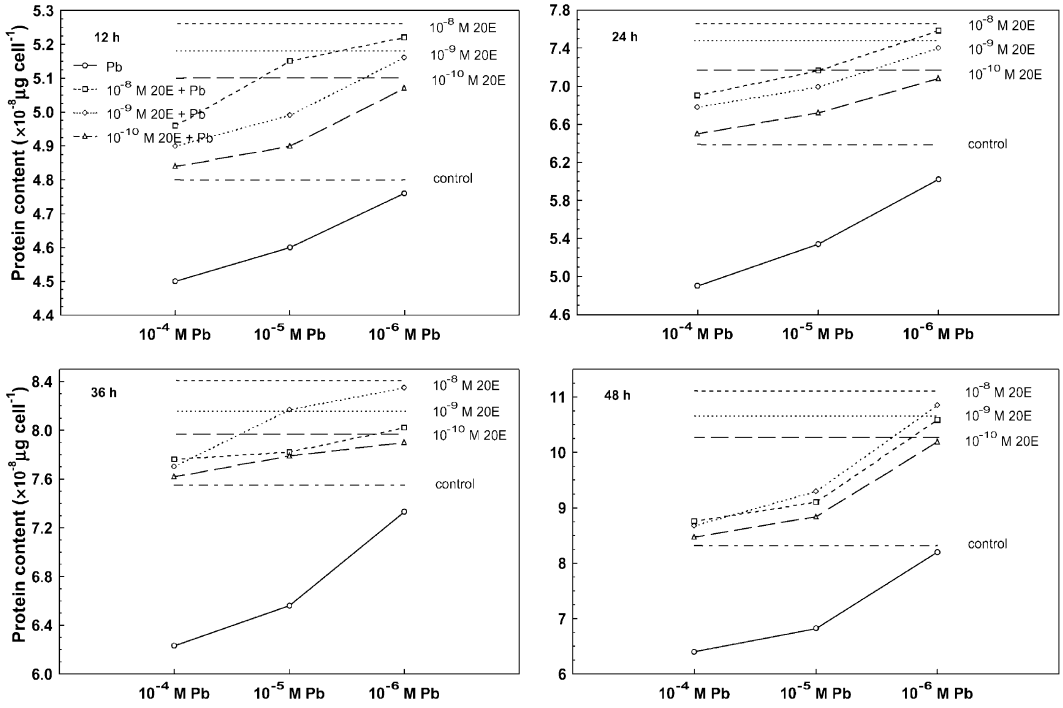

Fig. 7. Effect of lead in the absence or presence of 20E on the protein content in *C. vulgaris* cells. Data are means±SE of results from 15 replicate samples. Error bars were smaller than the symbols for most points. Before treatment of *C. vulgaris* cultures, the protein content was established at 4.50×10^{-8} µg cell^{-1}.

3. Discussion

Microalgae can be used to remove toxic heavy metals from waste streams due to the well-known ability of microorganisms to accumulate metal cations. Algae have provided useful model systems for investigating the processes and associated chemical and biological factors regulating cellular metal accumulation and resultant physiological effects. In practice, metal accumulation by plants is restricted by metal bioavailability in the environment. The use of chemical chelates, which remove metals from their binding sites in the water, was once proposed as a solution to this problem. However, although the accumulation of metals by plants can be enhanced by the addition of chemical chelates, these compounds can purge the metals too efficiently, resulting in pollution of the water table (Harding and Whitton, 1976; Rai, 1981; Skowronski and Przytocka-Jusiak, 1981; Sandau, 1996; Shubert, 1984; Vymazal, 1990; Crowder, 1991; Ilangovan, 1992; Rachlin and Grosso, 1993; Wilde and Benemann, 1993; Volesky and Holan, 1995; Vilchez et al., 1997).

Many studies on heavy metals polluted waters have revealed that metal pollution decreases algal diversity, productivity and alters algal species composition. However, there are also many reports concerning occurrence of several algal (Harding and Whitton 1976; Fahmi, 1982; Foster, 1982; Takamura et al., 1989; Gupta and Chandra, 1994) and cyanobacterial species (Vymazal, 1990) which are tolerant or resistant to cadmium, copper, lead or zinc. Lead was found to be accumulating by aquatic plants and algae (Gupta and Chandra, 1994; Bilgrami and Kumar, 1997; Bajguz, 2000a, 2002a; Pawlik-Skowronska, 2002, 2003; Tsuji et al., 2003) and many disturbances caused by lead in the algal ultrastructure and metabolism were reported (Rai, 1981; Kessler, 1986; Rachlin and Grosso, 1993; Poskuta et al., 1996; Bajguz, 2000a, b).

No experiments concerning the influence of ecdysteroids upon lead-treated plants have been conducted so far. The effects of 20E mixed with lead on the growth (Fig. 1) and the levels of lead, PC, chlorophyll, sugar and protein (Figs. 2, 3, 5–7) in *C. vulgaris* cells have been studied. Treatment of cultured *C. vulgaris* cells with 10^{-6}–10^{-4} M lead inhibits their growth and the cellular content of chlorophyll, sugar and protein during the 48 h of cultivation. Heavy metals have been found to decrease the total chlorophyll and sugar content, and the chlorophyll *a*/*b* ratio, and decrease the chlorophyll/carotenoid ratio in plants. Heavy metals inhibit the biosynthesis of chlorophyll pigments and enzymes involved in this process (Takamura et al., 1989; Poskuta et al., 1996). The most inhibiting influence upon the number of *C. vulgaris* cells was shown by lead at 10^{-4} M between the 12th and 36th h of cultivation. Lead at 10^{-6} M has a slightly inhibitory effect on the growth of *C. vulgaris*.

Ecdysteroids can be considered as brassinosteroid-related compounds or vice versa. Brassinosteroids exhibit structural similarities with ecdysteroids in that they both contain the entire cholesterol skeleton with the complete side chain. Nevertheless the carbon skeleton of ecdysteroids differs from cholesterol in the position of the double bond, e.g. Δ^5 in cholesterol, Δ^7 in ecdysteroids. The existence of two hydroxyl groups in the A-ring and two in the side chain makes brassinosteroids and ecdysteroids similar. However, the similarity between brassinosteroids and ecdysteroids is not so obvious, because many differences exist in the A/B ring junction (*trans* stereochemistry of brassinosteroids vs. *cis* of ecdysteroids) and position of the hydroxyl group in the side chain. It leads to striking differences between the spatial shape of both types of steroid molecules and certainly may determine their biological properties (Lafont, 1998; Bajguz and Koronka, 2001; Dinan, 2001; Bajguz and Tretyn, 2003). Brassinosteroids may act through a mechanism similar to that of animal steroid hormones. This action is regulated via a soluble receptor/ligand complex which binds to nuclear or cytoplasmic sites to regulate the expression of specific genes (Thummel and Chory, 2002). If ecdysteroid action is mediated by a membrane receptor, it would not seem to be a brassinosteroid receptor, since the nature of the response of *C. vulgaris* to ecdysteroids and brassinosteroids is different, even if both are stimulatory of all the biochemical parameters discussed here; brassinosteroids act more rapidly and the response is more intense. On the basis of the action of the brassinosteroid biosynthesis inhibitor brassinazole, the existence of brassinosteroids in *C. vulgaris* is probable (Bajguz and Asami, 2004). Furthermore, *C. vulgaris* has not yet been examined for its ecdysteroid content. Brassinosteroids and ecdysteroids participate in the growth of plant tissue in the processes of transcription and translation. It was demonstrated that the activation of the growth of plant tissue and higher levels of RNA and DNA polymerase is manifested by the increase of the content of DNA, RNA and proteins (Bajguz and Czerpak, 1998; Bajguz, 2002b; Bajguz and Dinan, unpubl.).

The decreased growth and the content of chlorophyll, sugar and protein in *C. vulgaris* cells treated with lead was restored by the co-application of 20E (10^{-10}–10^{-8} M). *C. vulgaris* cells treated with both lead and 20E showed growth promotion (Fig. 1). Growth-promoting effect of 20E increased in a concentration-dependent manner in lead-treated *C. vulgaris* cells. The levels of chlorophyll, sugar and protein are increased in 20E-treated *C. vulgaris* cells (Figs. 5–7). Optimal stimulation is observed at 10^{-8} M 20E. At concentrations lower than 10^{-8} M the stimulation gradually reduces, but is still significantly above control. Although the growth and metabolism of *C. vulgaris* cells treated with 20E and lead was lower than in the case of 20E application. 20E

stimulates growth of *C. vulgaris* cells over a wide concentration range (10^{-16}–10^{-7} M) (Bajguz and Dinan, unpubl.). High concentrations ($>10^{-6}$ M) are cytotoxic. It has a stimulatory effect on growth and metabolism of the algae between the 3rd and 9th day of cultivation. Optimal stimulation is observed at a concentration of 10^{-9} M at the 7th day of cultivation. The phase of rapid growth of *C. vulgaris* was characteristic for the cells treated with 20E or not (control) between the 3rd and 7th day of cultivation. In turn, the phase of growth stagnation occurred between the 7th and 9th day. The cellular content of nucleic acids, protein, sugar, organic and inorganic phosphorus, chlorophylls, phaeophytins and carotenoids are all stimulated by 20E treatment, when compared to non-treated control cells. Algal cells treated with 20E contain approximately 2–3 times more analyzed biomolecules than control cells. 20E stimulates enlargement of the algal cells, which is associated with asynchronous cell division. Growth of *C. vulgaris* is manifested as an increase in the size of each individual cell followed by division into daughter cells. The size of mother and adult daughter cells is normally the same. However, the adult *C. vulgaris* cells treated with 20E increased in size. The continuous occurrence of ecdysteroids in the medium effects an increase in cell diameter and an acceleration of metabolic processes in unicellular alga. Understanding of the molecular mechanisms of this control is still unclear. It is correlated with the acceleration of PC synthesis by 20E and a decrease in lead content in *C. vulgaris* cells.

Production of PC is a constitutive mechanism for coping with elevated toxic metal concentrations. These thiol-containing peptides are produced in cells of plants, algae and fungi exposed to some heavy metals like cadmium, copper, lead, zinc, and other. PC, which plays an important role in heavy metal homeostasis and detoxification, are also considered as biomarkers of metal toxicity in higher plants (Rauser, 1995; Cobbett, 2000; Cobbett and Goldsbrough, 2002). The relationship between metal ion concentration inside the cell and PC suggests that the mechanism of PC synthesis is controlled by the metal entered inside the cell (Fig. 4). The ratio of SH groups to metal ions in cell allows us to see whether there is sufficient content of PC to chelate the total pool of metal. When the ratio is between 1:1–4:1 the total pool of metal has a chance to be detoxicated by PC. On the other hand, when the ratio is lower from 1:1 the free metal ions are found in cytoplasm and they show toxic effect on the algal growth (10^{-4} M lead at the 1–3 h). The content of PC demonstrated linear increase at the first hours of metal action. The lengthening of the time of exposition on lead is accompanied by an increased level of PC (the ratio of PC:Pb was even >30:1; Fig. 4). So, algae are capable to synthesize the exact concentration of PC necessary to bind intracellular metal. This should be the necessary requirement of an effective detoxification mechanism. The increase in PC production in algal cells in response to that in intracellular heavy metals content suggests that the PC level might be an indicator of toxic metal bioavailability under complex environmental conditions. Algae are able to bind high amounts of heavy metals from the surrounding water, and they respond to the intracellular accumulated metal by synthesizing PC (Skowronski and Przytocka-Jusiak, 1981; Rachlin and Grosso, 1993; Pawlik-Skowronska, 2002, 2003; Tsuji et al., 2003). Lead is an environmental contaminant, which, at high concentrations, exerts adverse effects on morphology, growth and photosynthetic processes of chlorophytes (Christensen et al., 1979; Takamura et al., 1989; Poskuta et al., 1996; Bajguz, 2000a, b; Pawlik-Skowronska, 2002). The interaction of 20E with lead showed a weaker accumulation of this heavy metal by algal cells than the cultures treated with lead alone (Figs. 2–4). 20E reduced the toxicity of lead and the growth recovered to the level of cells treated with 20E alone. The content of PC in *C. vulgaris* cells was proportional to the concentration of 20E and lead. An increase in lead concentration caused an increase in PC content in *C. vulgaris* cells.

The influence of brassinosteroids on the accumulation of heavy metals has been studied for alga *C. vulgaris* (Bajguz, 2002a) and different agricultural plants such as barley, tomato, radish, sugar beet (Khripach et al., 1999). It was found that the application of brassinosteroids significantly reduced the metal absorption. For example, the content of lead was more than 50% lower than in lead-treated cultures. It was correlated with an acceleration of PC synthesis in *C. vulgaris* cells by brassinosteroids (Bajguz, 2002a).

Finally, lead blocked algal growth and metabolism and this blockage was reversed by co-application of 20E with lead. It suggests that 20E has a positive effect on the growth and metabolism of *C. vulgaris* cells against lead stress. Furthermore, 20E may exist as a stimulant of purification of algal cells from lead by the acceleration of PC synthesis. These facts indicate that ecdysteroids have an anti-stress activity.

4. Experimental

All reagents used in analytical methods were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

4.1. Plant material and growth conditions

Chlorella vulgaris Beijerinck (Trebouxiophyceae) was grown under controlled conditions at 25 ± 0.5 °C, 16-h photoperiod (photon flux of 50 μmol $m^{-2}s^{-1}$ at the surface of the tubes). Permanent synchronous growth

was established according to the method of Pirson and Lorenzen (1966). The culture medium used was Knop's medium in the following nutritive solution: $4.94\cdot10^{-3}$ M KNO_3, $2.12\cdot10^{-3}$ M $Ca(NO_3)_2\cdot4H_2O$, $1.47\cdot10^{-3}$ M KH_2PO_4, $6.09\cdot10^{-4}$ M $MgSO_4\cdot7H_2O$, $3.70\cdot10^{-5}$ M $FeSO_4\cdot7H_2O$, $4.85\cdot10^{-5}$ M H_3BO_3, $1.01\cdot10^{-5}$ M $MnCl_2\cdot H_2O$, $2.56\cdot10^{-6}$ M NH_4VO_3, $6.96\cdot10^{-7}$ M $ZnSO_4\cdot7H_2O$, $8.09\cdot10^{-8}$ M $(NH_4)_6Mo_7O_{24}\cdot7H_2O$. The pH of the medium was adjusted to 6.8 with 1 M NaOH. The *Chlorella* cells were cultured in an Erlenmeyer flask (500 ml) containing 250 ml of medium, and shaken at 100 rpm in a rotary shaker.

4.2. Phytoecdysteroid and lead

With regard to previous papers (Bajguz, 2000a, b, 2002a; Bajguz and Koronka, 2001) the concentrations 10^{-10}–10^{-8} M 20E and 10^{-6}–10^{-4} M lead were selected. 20E was prepared as 10^{-6} M ethanolic stock solutions and stored at −20 °C. The appropriate amount of 20E stock solution for the strongest solution was transferred directly into culture medium and weaker solutions were prepared by serial dilution. Equal amount of ethanol was added to the controls. The final ethanol concentration in the culture media did not exceed 1% (v/v), and this concentration did not affect the growth of algae. Sterile solutions of $Pb(NO_3)_2$ were added to the algal cultures to obtain the required concentration of lead in the growing solution.

4.3. Number of cells

Number of cells was determined by direct counting of cells in the growth medium using the Bürker chamber.

4.4. Cellular lead content

The content of lead was determined by the aid of an atomic absorption spectrometer (Carl Zeiss Jena, Germany) with background compensator and an air-acetylene flame. For the determination of the total lead accumulation, the algae were first collected by centrifugation (3300 *g*, 15 min, 20 °C) of 25 ml culture samples and then the algal pellets were washed in distilled water. Then the samples were dried at 105 °C to constant weight and dissolved in 65% HNO_3.

4.5. Phytochelatins determination

Total PC content was detected by HPLC method with postcolumn reaction with Ellman's reagent according to Tukendorf and Rauser (1990). The algae were first collected by centrifugation (3300 *g*, 15 min, 20 °C) of 10 ml culture samples and then the algal pellets were homogenized in a small mortar and pestle on ice with a quadruple volume of 0.1 M HCl. Homogenates were centrifugated (5000 *g*, 5 min, 4 °C) and obtained supernatants were used for chromatographic method.

4.6. Chlorophyll determination

For chlorophyll determination, the algae were first collected by centrifugation (3300 *g*, 15 min, 20 °C) of 10 ml culture samples and then the algal pellets were homogenized in methanol. The absorbance of the extract was measured at 653 and 666 nm. The amounts of chlorophyll $a+b$ present in the extract were calculated according to the equations of Wellburn (1994).

4.7. Sugar determination

For sugar determination, the algae were first collected by centrifugation (3300 *g*, 15 min, 20 °C) of 10 ml culture samples. Cellular sugar content was determined using the Somogyi (1954) method.

4.8. Protein determination

Measurement of protein content was done by extracting the algal pellet overnight in 0.1 N NaOH at 4 °C. Concentration of cellular protein was determined by the method of Lowry et al. (1951) with a protein kit calibrated with bovine serum albumin as the standard.

4.9. Replication and statistical analysis

Each treatment consisted of 3 replicates and each experiment was carried out at least 5-fold at different times. Experiments were replicated at the same time of the life-cycle of *C. vulgaris*. Analysis of growth and selected biochemical parameters was performed on the beginning and late phase of light period. It has been established to count normal cells in untreated and treated cultures. Minitab statistical package was used to carry out a one-way ANOVA. Significance was determined using *t*-tests and LSD values based on the ANOVA data.

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