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Organic Soil Amendments in Assisted Phytoremediation of Heavy Metals Contaminated Soil - Basic Phytotoxicity Markers

Organiczne dodatki we wspomaganej fitoremediacji gleb zanieczyszczonych metalami ciężkimi - podstawowe markery fitotoksyczności

Abiotic and biotic stressors induce a strong cellular response in plants, resulting in significant changes in plant cells metabolism. Knowledge in this area can help to develop very specific methods of stress decrease. For this purpose, it is necessary to find sensitive and accurate tools that will allow to estimate the level of stress in cells. The largest group of substances involved in stress response are proteins. The study of changes in the protein profile can yield the most answers concerning the different mechanisms of soil microflora tolerance and the adaptation of plants to unfavorable conditions. The knowledge of these mechanisms can significantly support the assisted phytoremediation of soils. The aim of the study was to investigate the effect of abiotic factors on plant stress response in *Lupinus luteus* grown on soil contaminated with heavy metals and amended with organic additives. The aim of the study was to determine the level of plant stress based on tests of plant stress proteins presence and a specific peroxidase activity in plants grown on soil contaminated with heavy metals (HMs). An acrylamide gel electrophoresis (SDS-PAGE) has been used in the study to separate specific proteins fractions (metallothioneins), accompanying stress factors. The results of peroxidase activity indicated that the organic soil amendments have an impact on reducing plant stress. The lowest dose of soil amendments reduced the amount of peroxidase by almost half in roots. This proves that adequate soil supplementation helps plants to tolerate stress. The SDS-PAGE analysis suggests that in the most stressful conditions the protein profile is significantly different from control and indicates additional small protein products in the range of 7÷20 kDa indicating, in accordance with the literature, presence of metallothioneins as response to plant stress. The applied methodology confirms that organic soil amendments reduced the level of HMs plants stress after organic amendments soil application.

Keywords: **SDS-PAGE, toxicity, bioremediation, organic soil amendments, heavy metals**

Introduction

Research involving harmful effects of heavy metals (HMs) gain on importance with growth of environment pollution and degradation of agricultural soil [1]. Intake of HM to plant cells is highly connected to qualities of the growth medium. Mobility of elements depends on pH, natural absorbent and binding compounds

and on ionic balance. For example acidic conditions promote ion mobility and research suggest most HM enter the cells in this state, using existing transporters in cell membranes [2]. Excess of HM is evident among other symptoms as slower growth, organ damage, chlorosis, withering and root shortening. It's a result of three general mechanisms: stimulation of ROS and free radicals production, binding of HM with active sites of enzymes and swapping of their important cofactors. HM also negatively impact water uptake leading to cell membrane damage and blockage of water passages. Along comes decrease in turgor, resulting in closing of stomata and stress due to lack of water. Intake of minerals is also obstructed or even completely blocked due to HM competing for transport routes [2-4].

Some plants exhibit increased tolerance for HM. However even those species suffer irreversible damage after exceeding limit doses. Usually plants cope with HM in one of two ways. First is blockade of intake by enabling mechanisms to stop usual transport passages. Exact mechanisms are various and include chelating elements in rhizosphere, decreasing membrane and cell wall permeability, binding of trace elements on cell walls and mycelium of mycorrhiza. The second strategy includes neutralization of ions and damage they induce by binding them with proteins and chelators in vacuoles and enzymatic repair of protoplast structures hurt by metals and ROS [2, 3, 5, 6]. Research of plant stress led to discovery of hundreds of proteins which expression changes in case of stress and so they are called stress proteins. Those substances have many different functions, sometimes highly specific, taking part in a specialized stress response. Exemplary groups of stress proteins, some of which are involved in many different scenarios are peroxidative stress proteins. They divide in two groups: enzymes and non-enzymatic antioxidants. Along enzymes we can differentiate those degrading ROS and also the ones responsible for reduction of glutathione and ascorbate. Group of non-enzymatic peptides consist of: ascorbate, antioxidant that exhibits affinity to O_2, H_2O_2, OH and singlet oxygen and peroxide radicals. In the cell ascorbate cooperates with glutathione in Halliwell-Asada cycle. Decrease of negative influence is aided by thioredoxin which reduces invalidly created disulfide bonds in oxidized proteins [2, 5, 7, 8].

Superoxide dismutase catalyze partitioning of superoxide radical to molecular oxygen or hydrogen peroxide. The latter is degraded by other enzymes, catalases and peroxidases. Catalases are responsible for dismutation of peroxide to water and oxygen. Ascorbate peroxidase reduces H_2O_2 by involving two molecules of ascorbate. Further reactions lead to production of glutathione disulfide (GSSG) out of ascorbate. GSSG is then regenerated to glutathione by enzyme called glutathione reductase. Peroxidase has higher affinity to H_2O_2 which suggests catalase has higher importance in deactivation of this compound in stress conditions [2, 7-10]. Resistance to HMs in plants is conditioned by presence of many groups of proteins and other substances which enable not only stopping of intake of harmful substances but also maintain proper concentrations of beneficial minerals. Many different proteins are responsible for transport and sequestration of ions. First of such protein group are so called chaperons which function is to transport ions to the place they are necessary, for example to embed them into enzymes. This way chaperons

support other proteins in their duty and limit production of ROS. Other ions, not necessary for current metabolism, are bound by various chelators. One example of such compounds are metallothioneins (MTs), which function is bind HMs ions and such complexes are stored in vacuoles. This compounds are well studied in case of animal and yeast cells where three classes were observed. I and II class are molecules created directly by gene expression and their function is to intercept redundant ions. It is speculated that metallothioneins have important function in regulation of ion concentrations, similarly to chapperons. III class are peptides synthetized without influence of mRNA. They are called phytochelatins and their function is to quickly bind metal ions. In form of such complex they are transported to vacuoles where ions unbind and phytochelatin returns to cytosol to connect with another ion. Thereby the tolerance to stress depends on tempo which complexes phytochelatin-metal are transported to vacuoles [2, 7-9].
In similar work on fish protein molecular weights of MT were shown as 11÷14 kDa [11] while in plant material it was estimated to be around 7 kDa [12]. The purpose of this research was to detect proteins of similar weight (7kDa) in plant material and to check whether occurrence of such products can be correlated with stress levels induced artificially by cultivating the plants on HM degraded soil.

1. Materials and methods

1.1. Soil and substrates characteristics

The study was conducted using *Lupinus luteus* grown on various soil treatments. Soil was collected from area of zinc and lead processing operation in south Poland (GPS 50.510397, 18.936439). In the investigated soil the high concentration of heavy metals such as Pb (1200 mg/kg), Zn (900 mg/kg) and Cd (14 mg/kg) were present [13]. Commercial peat soil mixture was used as a reference soil (control). Potting mixes were prepared by mixing contaminated soil from Miasteczko Śląskie and 10, 15 or 20% w/w ratio of cattle manure (CM) or horse manure (HM). The control (MS) soil (contaminated, 0% organic fertilizer amendments) was supplemented with sand in the same ratio as treatment to avoid “dilution” effect. The 10 seeds were planted in each pot and plants were grown in triplicates for a total of 24 pots. Experiment was carried out in a growth chamber at constant conditions: 16/8 hour day/night photoperiod at respectively temperatures of 21 and 18°C and ~100% humidity. After 28 days of growth in chamber, watering, the plants were harvested and inspected for abnormal growth. Roots were rinsed to remove remaining soil. Aboveground parts and roots were separated and after saving small quantity of fresh material the rest was frozen in –80°C for further analyses.

1.2. Protein extraction

From fresh plants biomass samples of 100 mg were weighted. All samples were prepared in three repetitions. To extract proteins the samples were macerated with

addition of 700 µl of extraction buffer (50 mM TRIS, 2 mM EDTA, 1 mM PMSF, 1 mM β-mercaptoethanol). Prepared samples were placed on shaker in 4°C at 200 RPM. After 2 hours samples were centrifuged for 25 minutes at 14 000 RPM at 4°C and then 500 µl of supernatant was transferred to new tubes. To that 1.5 ml of chilled acetone was added and samples were incubated in –20°C for 20 minutes and centrifuged at 14 000 RPM at 4°C for 15 minutes. Liquid was discarded and the remaining sediment was dried under laminar flow hood. Finally the solid was dissolved in 100 µl of storage buffer and frozen at –20°C.

1.3. Peroxidase activity measurement

To assay peroxidase activity frozen biomass was used. The protocol used was modified Flocco method [6]. 100 mg of sample was macerated with 3 ml of phosphate buffer. Samples were then centrifuged at 11 000 rpm for 5 minutes and 2 ml of supernatant was transferred to new tubes. To assay peroxidase activity 10 µl of obtained extract was added to 3 ml of guayacol solution and after addition of 10 µl of 30% hydrogen peroxide the sample was mixed and absorbance was immediately measured at $\lambda = 470$ nm. The reading was the highest value measured in 2 minutes period. Remaining extract was stored for further analysis at –20°C.

1.4. Lowry protein assay

To properly analyze peroxidase activity measurement of total protein was necessary. Assay was run according to modified Lowry method [14]. 700 µl of freshly prepared Lowry solution was mixed with 250 µl of plant extract and 250 µl ddH_2O. Samples were vortexed and incubated in darkness for 30 minutes. Fresh Folin reagent solution was prepared and 100 µl was added to each sample. Vortexed samples were again placed in dark room for 30 minutes and after that absorbance was measured at $\lambda = 750$ nm. To obtain result absorbance was compared to standard curve prepared on the basis of bovine albumin.

1.5. Bradford protein assay

To ensure same amounts of protein loaded on gel for SDS-PAGE each protein sample was assayed using Bradford method, dedicated for high protein range concentration [15]. First the Eppendorf Biophotomether was calibrated according to manufacturer's instructions using bovine albumin fraction. Samples were prepared by adding 5 µl of measured protein solution to 500 µl of Bradford solution and mixed thoroughly. After 5 minutes absorbance was measured and automatically calculated into result in µg protein/ml of sample. All samples were run in 3 repetitions.

1.6. SDS-PAGE

Protein samples were prepared by mixing 30 µl of sample with 10 µl of loading buffer (if necessary the concentration was adjusted by diluting sample with

ddH_2O according to Bradford assay results) and incubated in 95°C for 5 minutes. SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel) electrophoresis was carried out using 5% stacking and 10% separating gel. All buffers were prepared according to [16]. The 5 µl of Eurx Perfect Color was used as marker and samples were loaded in two repetitions after ensuring the protein concentration is around 200 to 300 ng/µl to avoid well overloading. Gels were run for 80 minutes at 200 V, 35 mA per gel (two gels were run at the same time). Next the gels were stained with Thermo Scientific PageBlue according to protocol using microwave oven. Stained gels were rinsed twice and left in water for the night and photographed the next day [16].

2. Results and discussion

2.1. Plants growth and development-germination index testing

Number of germinated and developed plants was compared with initial count of planted seeds (Fig. 1). Resulting numbers are shown as % of developed plants. Most plants grown properly on soil treated with 10% cattle manure and the least amount grown on 10% horse manure mix. For control (contaminated soil, MS) only 52.7% plants germinated while on reference soil with no additives the germination index reached 88.9%. This confirmed the negative effects of heavy metals on plants germination. For conducted study the improvement of germination index was increased for 10 and 15% of cattle manure addition. In the research of Jaskulak et al. [17] organic soil amendments also increased the germination index of *Sinapis alba* L. and *Robinia pseudoacacia*.

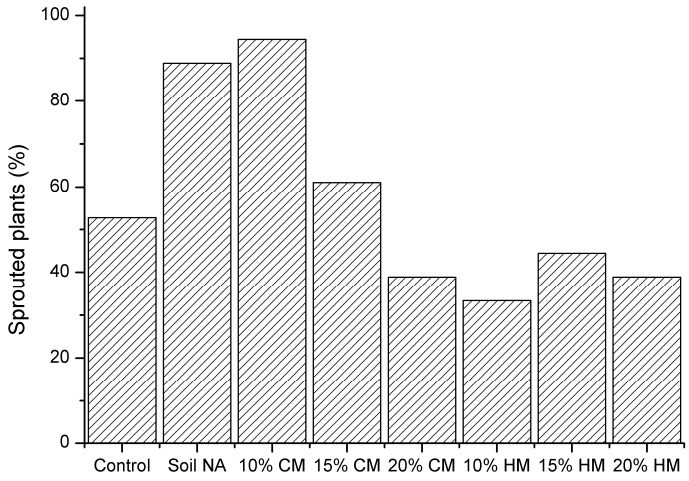

Fig. 1. Germination index of *Lupinus luteus*; Contol (contaminated soil without any amendments); Soil NA - reference soil with no additives, CM - soil treated with 10, 15, 20% cattle manure, HM - soil treated with 10, 15, 20% horse manure

Development of plants' organs differed in each of the treatment group. The highest and the most developed plants grown in peat soil (reference soil). Plants grown in contaminated soil with no additives (Control) were smaller and the roots were not longer than 5 mm. Plants grown in soil with additives were developed properly but were much smaller than ones grown in reference soil. Moreover, it was found that *L. luteus* grown in soil containing 10% cattle manure did not develop long roots but overall length of the plants was higher than plants grown in contaminated soil with no additives. Most similar to control were the plants grown in mix with 20% cattle manure. No chlorosis was observed on any of the plants (Fig. 2).

Fig. 2. Selected plants of *Lupinus luteus;* left to right: reference soil, contaminated soil with no additives, contaminated soil treated with 10% cattle manure, 20% cattle manure (photo by M. Jaskulak)

The investigated study confirmed the negative effect on plants growth under HMs concentration in soils. In the study of Wołejko et al. was also found the negative effect of white mustard fertilization with HMs contaminated sewage sludge [18] and the excessive amounts of Cd in seeds.

2.2. Protein assays

Lowry protein assay indicated that more proteins were detected in roots than in leaves. Between the treatments the differences were insignificant, with one exception, of 20% of cattle manure soil treatment (Fig. 3). This confirms that total protein assay does not give any information about the plats HMs stress, indicating the qualitative protein analysis as a suitable toxicity determination assay. Plant responses to HMs are present as a consequence of changes in protein expression. The qualitative and quantitative changes in proteins are a consequence of the expression of specific genes, which may not change the amount of total proteins content [19].

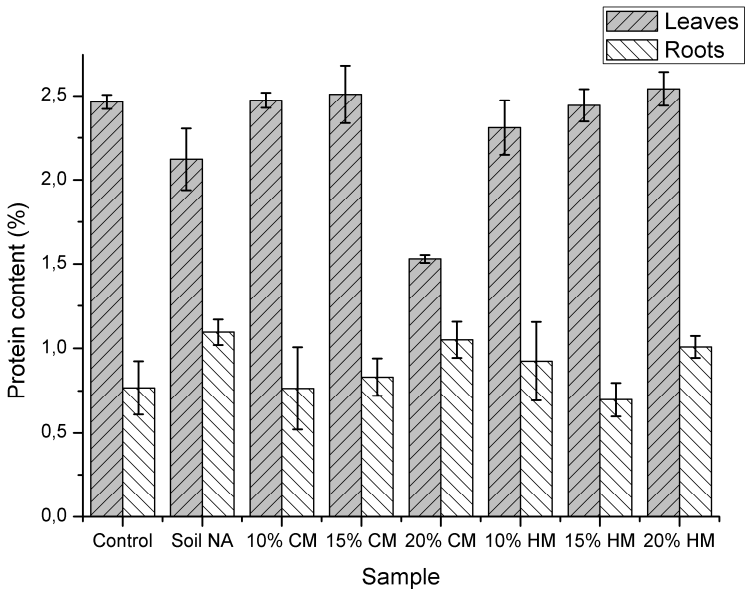

Fig. 3. Total protein content of *Lupinus luteus*; Control (contaminated soil without any amendments), Soil NA - reference soil with no additives, CM - soil treated with 10, 15, 20% cattle manure, HM - soil treated with 10, 15, 20% horse manure

2.3. Peroxidase assay

Peroxidase activity was calculated as units of enzyme per mg of total protein (U/mg protein), separately for roots and leaves. It was found that roots contained much higher peroxidase activity than leaves. The highest levels were detected in plants grown on contaminated soil (Control) with no additives. This confirms that roots were subjected to much higher level of oxidative stress caused by high heavy metal content in rhizosphere. Peroxidase activity in leaves were similar (without significant differences) in most of the different soil treatments, which suggests the root system acts as buffer zone and protects plants against transferring heavy metals from roots to higher parts of *L. luteus*. The direct exposition to HMs of plants tissues, especially metals in soils, is affecting the highest stress response mainly in such plants tissues as roots [17, 20]. Both cattle manure and horse manure had significant impact on lowering the peroxidase activity in plants (Fig. 4).

In the research of Jaskulak et al. the 5% difference in the quantity of the used soil amendment caused significant differences in peroxidase activity in the roots of *S. alba* L. and *R. pseudoacacia* L. [17]. Peroxidase activity is very sensitive tool for cadmium stress response in plants [5, 21]. Moreover as indicated in presented study, the peroxidase activity mainly in roots can be found as quick and effective method for assessing soil toxicity.

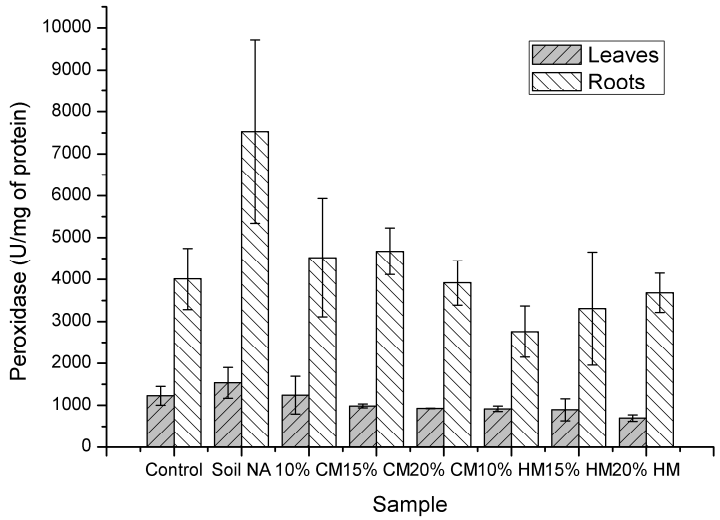

Fig. 4. Peroxidase activity as number of units of enzyme per mg of total protein; Control (contaminated soil without any amendments), Soil NA - reference soil with no additives, CM - soil treated with 10, 15, 20% cattle manure, HM - soil treated with 10, 15, 20% horse manure

2.4. SDS-PAGE

SDS-PAGE electrophoresis of leaf proteins proven similar results between organic fertilizer amended samples. There was no significant differences between different soil treatments with HM and CM (Fig. 5). SDS-PAGE of leaves proteins showed significant differences between treated and untreated samples, mainly between control (MS) and reference soil without additives (NA soil). Most proteins had size between 50 and 25 kDa (Fig. 5). For sample 3, i.e. contaminated soil, and samples 7 and 9, additional protein products may be noticed in the area of metallothioneins (MT) and phytochelatins between 7 and 20 kDa. These are proteins of 18, 17, 16, and 7 kDa, respectively, which proves the most stressful soil conditions (MS soil). The research of Chan et al. [12] also confirmed the metallothioneins presence under stress condition in plants. SDS Page technique can confirm the presence of metallothioneins in plants. However, the quantitative assessment can be conducted using RT qPCR technique [17]. In the case of root proteins (Fig. 6), the results are not so clear probably due to indicated protein damage. The roots' proteins have been exposed to much higher damage, and thus their fragmentation can still occur inside the cell. The most homogeneous result was obtained for the control (reference soil) (no visible clear bands), while for the other samples a larger number of different proteins were noted. The most different groups of proteins were recorded for sample No. 3, contaminated soil (MS) without any amendments. Compared to other products, additional products in the area of 18, 14, 13 and 9 kDa were also noted. This confirms high damage of specific proteins or just expression of new ones as the effect of HMs soil contamination. Amini et al. [3] also detected repeated protein pattern changes in tomato under in vitro salt stress.

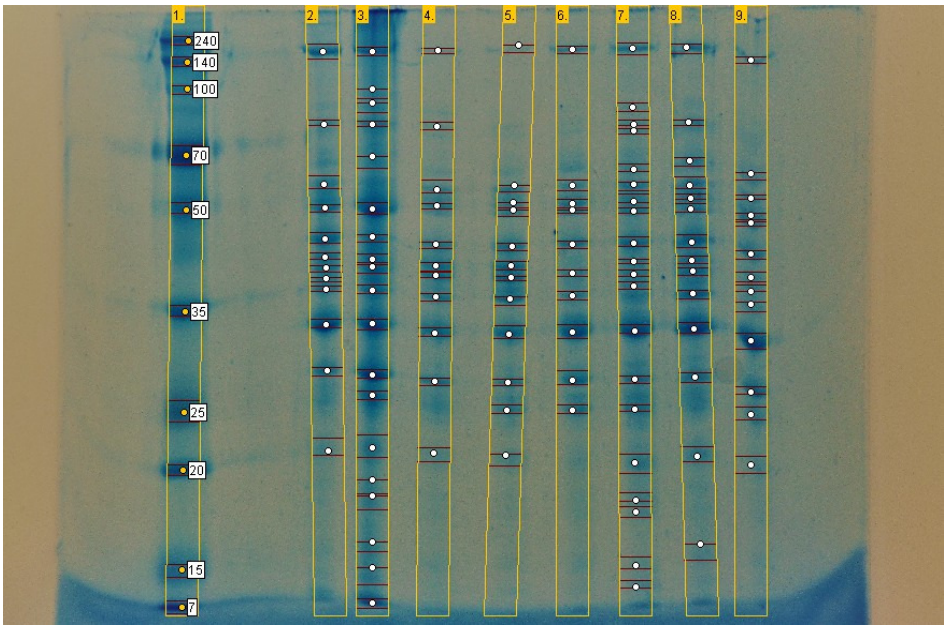

Fig. 5. Leaves proteins gel image; gel showing the different metallothionein band intensities; from the left: (1) marker with marked weights in kDa, (2) control (reference soil, NA), (3) soil contaminated without additives (MS), (4) 10% CM (cattle manure), (5) 15% CM, (6) 20% CM, (7) 10% HM (horse manure), (8) 15% HM, (9) 20% HM

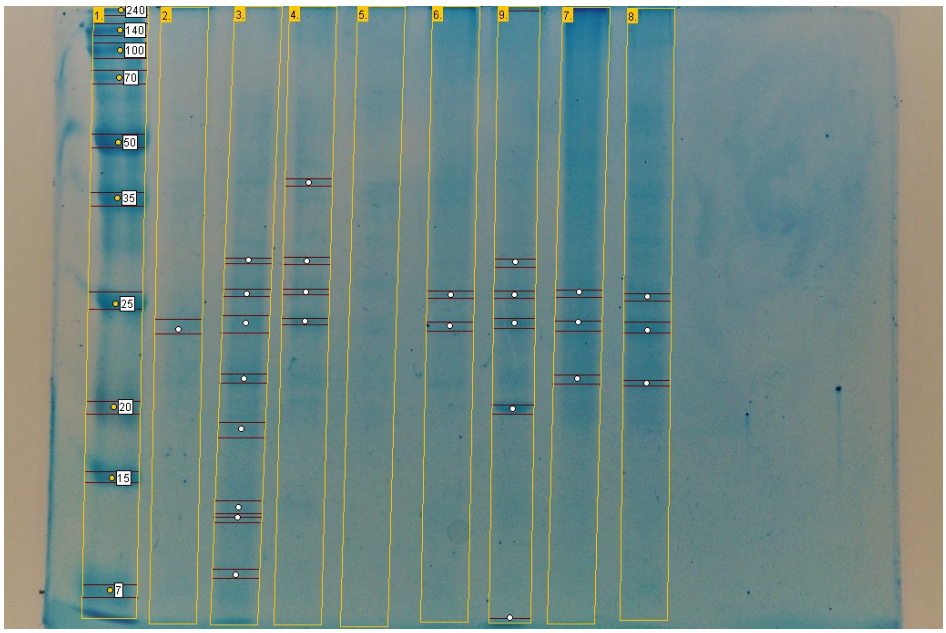

Fig. 6. Roots proteins gel image; gel showing the different metallothionein band intensities; from the left: (1) marker with marked weights in kDa, (2) control (reference soil, NA), (3) soil contaminated without additives (MS), (4) 10% CM (cattle manure), (5) 15% CM, (6) 20% CM, (9) 20% HM, (7) 10% HM (horse manure), (8) 15% HM

Conclusions

Analysis of metallothionein (MT) proteins by SDS-page technique can be used as reliable and cheaper method than the study of gene expression marker. However it needs to be confirmed in species other than *Lupinus*. Technique may be an useful in plant stress evaluation and indirectly it can indicate the condition of soil. The use of organic additives on contaminated soils create soil conditions eliminating the formation of biomarkers of plant stress - MT metallothionein proteins, detectable using SDS-page technique. In plants grown on soils contaminated without organic additives, the presence of stress proteins with low mass in the range of 7÷20 kDa (phytochelatin and metallothionein proteins) is observed.

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Streszczenie

Abiotyczne i biotyczne czynniki stresogenne wywołują silną odpowiedź komórkową u roślin, powodując istotne zmiany w metabolizmie komórek roślinnych. Pogłębienie wiedzy w tym zakresie może przyczynić się do opracowania metod zmniejszania reakcji stresowych roślin, a tym samym może zwiększyć odporność roślin na niekorzystne warunki glebowe. Konieczne jest więc stworzenie precyzyjnych narzędzi do szacowania poziomu stresu w komórkach. Największą grupę substancji zaangażowanych w reakcję na stres u roślin stanowią białka. Badanie zmian w profilu białkowym może dostarczyć odpowiedzi dotyczących poznania mechanizmów tolerancji mikroflory glebowej i przystosowania roślin do niekorzystnych warunków. Znajomość tych mechanizmów może w znacznym stopniu wpływać korzystnie na wspomaganą fitoremediację gleb.

Celem pracy było zbadanie wpływu czynników abiotycznych na odpowiedź stresową roślin *Lupinus luteus,* uprawianych na glebie zanieczyszczonej metalami ciężkimi i wzbogaconej glebowymi dodatkami organicznymi. W pracy badano poziom stresu roślinnego na podstawie testów obecności roślinnych białek stresowych i aktywności peroksydazy w roślinach uprawianych na glebie zanieczyszczonej metalami ciężkimi (HM). W badaniu wykorzystano akryloamidową elektroforezę żelową (SDS-PAGE) do rozdzielenia frakcji białek roślinnych (metalotionein), towarzyszących czynnikom stresowym.

Wyniki pomiaru aktywności peroksydazy wskazują na zmniejszenie stresu roślinnego, po wprowadzeniu do gleby zanieczyszczonej metalami dodatków organicznych. Najniższa dawka zastosowanych organicznych dodatków glebowych zmniejszyła aktywność peroksydazy o połowę w tkankach korzeni roślin. Dowodzi to, że odpowiednia suplementacja gleby pomaga roślinom tolerować stres. Wyniki analizy SDS-PAGE sugerują, że w najbardziej stresujących warunkach profil białka znacząco różni się od kontroli i wskazuje na pojawie-

nie się dodatkowych, niskocząsteczkowych produktów białkowych w zakresie 7÷20 kDa. Zgodnie z dostępną literaturą, wyniki wskazują na obecność metalotionein w odpowiedzi na ekspozycję roślin na metale ciężkie. Przeprowadzone badania potwierdzają, że wprowadzone do gleby zanieczyszczonej metalami ciężkimi dodatki organiczne, zmniejszyły wskaźniki stresu roślinnego.

Słowa kluczowe: SDS-PAGE, toksyczność, bioremediacja, organiczne dodatki glebowe, metale ciężkie