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# CHLOROPLAST STRUCTURAL AND FUNCTIONAL CHANGES AS BIOMARKERS OF HEAVY METAL CONTAMINATION

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The aim was to confirm the hypothesis of possibility to use the chloroplast structural and functional changes in higher plants as biomarkers to assess heavy metal contamination.

Chloroplast ultra-structural changes of Pisum sativum L were detected using the transmission electron microscopy. This work deals with studies of chloroplast structure responses to a high content of copper (250 μmM) and zinc (400 μmM). Data on changes in the structure of chloroplasts in particular, heterogeneity in the grain thylakoid packing, increase of interthylakoid gaps and thickness of chloroplast grain thylakoids in comparison with controls were obtained. The results of studies on structural and functional chloroplasts changes offer challenges for their use as markers for an early diagnostics of abiotic stress effects and in biotechnological studies to produce novel advanced varieties of crops resistant to stress.

***Key words:*** *Pisum sativum* L., chloroplast changes, heavy metal contamination, biomarkers.

Industrial emissions are a powerful factor that affects the vegetation cover development. Air, water and soil pollution by abnormal concentrations of chemicals has a direct influence on the plant photosynthetic apparatus. Effects of heavy metals (HM) excess concentrations on plants result in anatomical and morphological changes, physiological and biochemical disturbances. Excess of heavy metals negatively impacts cell organelles, changing their structure and characteristics.

In many regions of Ukraine environmental pollution arouses a strong interest in studies of heavy metal as a stress factor and finding mechanisms for organism protection against their toxic effects. Methods of bioindication are becoming increasingly important for the environmental conditions monitoring. Thus, for example, plant indicators are used to assess mechanical and acid composition of soils, their fertility, moisture content and salinity.

Based on the literature data, algae (*Prasinocladus marinus, Phaeodactylum tricornutum*) are known to be formerly used as indicators to assess a degree and pattern of contamination with heavy metals, such experiments using plants have not yet been conducted.Indication methods of heavy metals using biological objects are today very urgent since they have a low cost and because of an increasing man-made environmental pollution. Industrial emissions are a powerful factor that affects the vegetation cover development. Abnormal concentration chemical pollution has a direct impact on the plant photosynthetic apparatus.

According to Reimers classification, metals are referred as heavy when their density is higher than 8 g/cm$^3$, among them are — Pb, Cu, Zn, Ni, Cd, Co, Sb, Sn, Bi, Hg [1]. Excessive heavy metals concentrations in plants result in anatomical and morphological changes, in physiological and biochemical processes disturbances. Redundancy of heavy metals has a negative influence on organelle of cells by changing their structure and properties.

Trace amounts of some heavy metals ($Me^{2+}$) are required for plant growth and development.

Copper and zinc ions are in particular involved in the photosynthetic apparatus formation: plastocianine electrons

transporter contains copper while zinc is a part of plastocyanin, and zinc — superoxide dismutase and carbonic anhydrase. However, high concentrations of these metals in water or soil disturb many plant physiological processes; interrupt transportation functions, and various plant organs show destructive processes associated with an oxidative stress [2], there are also observed ultra-structural changes in the chloroplast membrane system [3]. Since plants are able to accumulate metal ions in cell envelopes and vacuoles of various tissue and organ cells, a substantial quantity of $Me^{2+}$ is excluded from an active metabolism and their concentration in various cell compartments may differ by several orders.

Thereby, the interpretation of physiological and structural changes in plant leaf organelles developing in conditions of an excessive $Me^{2+}$ content in soil requires additional studies because the observed effects may be caused either by an increasing $Me^{2+}$ concentration in the leaf or by dehydration and/or oxidative damages of the plant as a whole.

Most of metals enter the cell as cations (for example, $Zn^{2+}$, $Cu^{2+}$) as a result of an ordinary diffusion, and also via special transport systems [2]: CPx-type ATPases transporting essential and non-essential metals along the plasmatic cell membrane; cation/H+ antiporters; transport proteins of the NRAMP families (natural resistance associated macrophage proteins) [4] and CDF (cation diffusion facilitors) involved in the transportation of ions of Zn. $Zn^{2+}$, $Co^{2+}$, $Mn^{2+}$ и $Cd^{2+}$, and also the ZIP family proteins (Zinc-regulated transporters, Iron-regulated transporter-like Proteins) transporting ions of $Fe^{2+}$, $Mn^{2+}$, $Cd^{2+}$ и $Zn^{2+}$ [4]. These proteins-transporters were found in vacuole membranes and in chloroplast enclosures [2–6]. The higher HM content causes a sharp increase in plasmolemma permeability and, consequently, ion misbalance, leads to the loss of turgor and inhibition of the cell metabolism [2].

Mechanisms of cell protection against a HM abundance have been studied partially and include HM adsorption by internal and external enterosorbents, vacuolar compartmentation, activation of transporters exporting HM from cells, induction of proteins-chaperones [7]. Today, the mechanisms of HM effects on the plant structural components where photosynthesis occurs, namely on the chloroplasts membrane system, i.e. on the photosynthetic apparatus, remain studied insufficiently. The chloroplast internal membrane contains grana thylakoids and stroma thylakoids. Near the other components of the chloroplast membrane there is an important enzyme of their functioning, carbonic anhydrase (CA). The enzyme CA plays an important role in photosynthetic plant activities and also contributes to $CO_2$ transport facilitation and reduction of diffuse resistance to intracellular $CO_2$ transfer. It is known that carbonic anhydrase function blocking results from action of HM ions particularly, $Zn^{2+}$ and $Cu^{2+}$. Also proceeding from that, it can be assumed that carbonic anhydrase activity reduction may be a direct or indirect cause of photosynthesis disturbances. The aim of our studies was to estimate HM effects on the photosynthetic apparatus in pea seedling leaves.

14-day leaves of pea seedlings were used in our experiments. The leaf blades of *Pisum sativum* L. were soaked at 22 °C and lighting of 15 μM quanta/( $m^2$×s) for 2.5 days in Petri dishes in versions: in distillates, 250 μmM $CuSO_4$ or 400 μmM $ZnCl_2$. From leaves exposed to CA inhibitor solutions there were cut out 2–3 cm long segments. The preliminary fixation was done in conditions of sample vacuum infiltration in 1% glutaraldehyde on 0,1 M phosphate buffer (pH 7,0) at the room temperature. Samples fixation was carried out with 2,5% glutaraldehyde on 0,1 M cacodylate buffer, pH 7.6 for 4 hours at 4 °C. After washing in the same buffer (2 times for 20 min), the material post-fixation was conducted in solution of 1% $OsO_4$ on 0,1 M cacodylate buffer, pH 7,6, for one night at 4 °C.

A dehydration was done by sample washing in ethyl alcohol at increasing concentrations and in acetone. Then the material was soaked in epoxide resins and acetone mixture, poured in Epon-Araldite mix resin and transferred to thermostat for polymerization at 60 °C. Ultra-thin cell sections were obtained using ultramicrotome LKB-IV (LKB, Sweden). Sections were stained with uranyl acetate and potassium permanganate mixture (1:1) for 15 min in darkness. Ultra-thin sections were studied and filmed using transmission electron microscope JEM-1300 (JEOL, Japan). Preparation sections images were filmed by film for electron microscopy EB19H (AGFA, Belgium). After processing to analyze chloroplasts pictures and their segments morphometrically, the negatives were scanned. Pictures were made using the computer software Adobe Photoshop 7.0 and Corel Photo-Paint 11. 30–39 grana thylakoids enlarged ×10 000, or ×15 000, and ×100 000

**Thylakoid thickness and width of interthylakoid gaps**

| Variant | Control | 250 μmM $Cu^{2+}$ | 400 μmM $Zn^{2+}$ |
|---|---|---|---|
| Thylakoid thickness, nm | 4.7 ± 0.55 | 5.21 ± 0.55* | 8.46 ± 0.86* |
| Width of interthylakoid gaps, nm | 3.12 ± 0.50 | 3.43 ± 0.23 ** | 4.37 ± 0.69** |

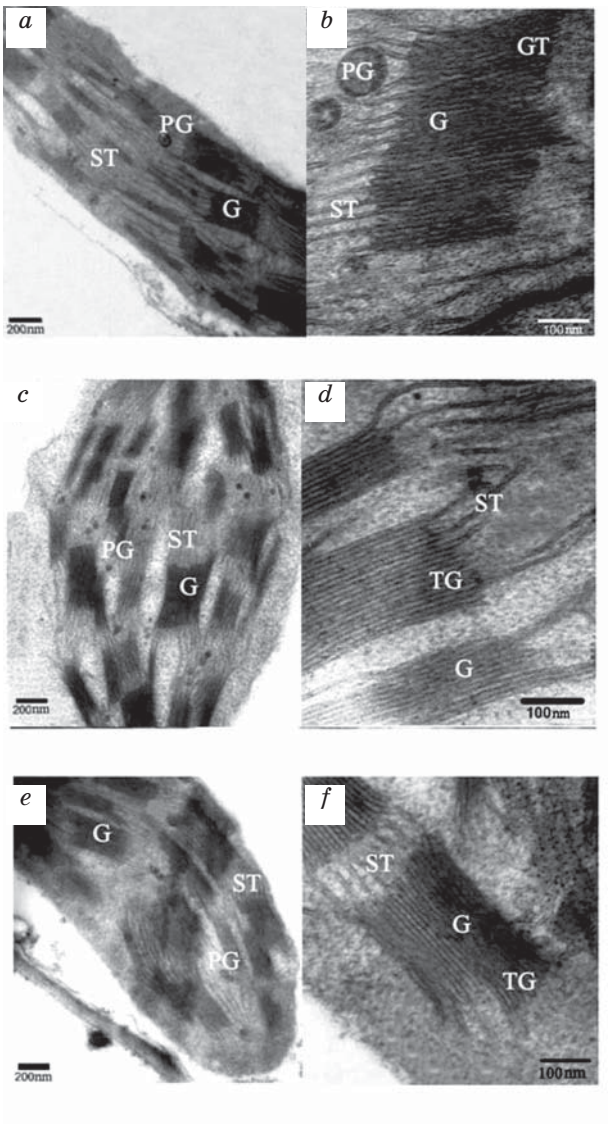
*Note.* * — $P < 0.05$ relative to control (250 μmM $Cu^{2+}$); ** — $P < 0.05$ relative to control (400 μmM $Zn^{2+}$).

were analyzed for each experiment version. The thylakoid dimensions on section pictures were measured using the free software UTHSCSA ImageTool 3.0. Statistic methods of data processing were also applied. The reliability of average values difference between test and control versions was evaluated by means of t-Student criterion. The difference was regarded statistically reliable at $P \le 0{,}05$.

Table shows data on calculation of thylakoid thickness and width of interthylakoid gaps.

The picture (Fig., *a*) presents chloroplast segments from control version showing clear-cut individual grana, plastoglobules, and thylakoids of stroma. The significantly enlarged picture presents an intact granum that consists of 42 thylakoids at the terminal segments of which there are attached stroma thylakoids (Fig., *b*). Under exposure to $Cu^{2+}$ (Fig., *c*, *d*), with a preserved granum general structure and uniform thylakoid packing in grana, the thylakoid of grana thickness exceeded that of control by 11%, while interthylakoid gap thickness — by 10%. Following the chloroplast treatment with $Zn^{2+}$ (Fig., *e*, *f*) there were observed some heterogeneity in grana thylakoid packing, granum structure changes that resulted in increase of thylakoid gaps by 14%, grana thylakoid thickness also increased by 18% as compared to control.

Chloroplast ultrastructure disturbances involving HM are one of the important causes of plant pigment content reduction and decrease in photosynthesis intensity [8]. Chloroplast membranes were found to contain proteins-transporters Zn/Cd that is an evidence of HM possible occurrence in these organelles [6] and, consequently, their direct effect on the chloroplast ultrastructure organization [3, 9, 10]. The photosystem II (PS II) is the most sensitive to metal ions [11]. Variation in the response center proteins structure is assumed to be the reason why PS II activity decreases when heavy metals occur in high concentrations [12]. There are available literature data indicating that heavy metals have a direct impact on electros transport in




**Fragments of chloroplasts:**
*a*, *b* — control (HC ×30 000, ×100 000);
*c*, *d* — $Cu^{2+}$ (×15 000, ×100 000);
*e*, *f* — $Zn^{2+}$ (×15 000, ×100 000);
G — grain;
GT — grana thylakoids;
PG — plastoglobules;
ST — stroma thylakoids

photochemical reactions [11]. High metals concentrations reduce $CO_2$ assimilation intensity both as a result of a direct action of their ions on some photosynthetic reactions and due to an indirect influence on other physiological processes. Insignificant changes in indices of thickness increase of thylakoids and interthylakoid gaps as compared to the spinach results we obtained previously [13, 14] may be related to the fact that heavy metal ions do not accumulate in chloroplasts despite the fact that their membranes were found to have Cd/Zn transporting ATPase, indicating that metals are probably delivered to these organelles [6]. Chloroplasts are able to accumulate heavy metals in protoplasts i.e. when plant cells have no cell wall [15].

Thus, heavy metals can affect photosynthesis that causes a reduction in photosynthetic pigments content and in carbonic anhydrase activity, which breaks the ultrastructure. The results obtained are important for understanding the mechanisms of heavy metals toxic effects, estimation of plant potential to adapt to unfavorable factors of the environment and may be used in solving the problems of the theory of plant resistance to extreme factors action.

Results of studies on chloroplast structural and functional changes may be used as markers in early diagnostics of abiotic stress effects, namely heavy metals, and also in biotechnological researches to produce novel, advanced technology crops resistant to stresses.

## REFERENCES


1. *Nikitin A. T.* Ecology, nature protection, environmental safety. *Moskva: MNEPU*. 2000, P. 648. (In Russian).
2. *Bertrand M., Poirier I.* Photosynthetic organisms and excess of metals. *Photosynthetica*. 2005. 43 (3), 345–353.
3. *Carrier P., Baryla A., Havaux M.* Cadmium distribution and microlocalization in oilseed rape plants (*Brassica napus*) after long-term grown on cadmium-contaminated soil. *Planta*. 2003, V. 216, P. 939–950. doi: 10.1007/s00425-002-0947-6.
4. *Techau M. E., Valdez-Taubas J., Popoff J. F., Francis R., Seaman M., Blackwell J. M.* Evolution of differences in transport function in slc11a family members. *J. Biol. Chem*. 2007, V. 282, P. 35646–35656. doi: 10.1074/jbc.M707057200.
5. *Pedas P., Husted S.* Zinc transport mediated by barley ZIP proteins are induced by low pH. *Plant Signal. Behav*. 2009, 4 (9), 842–845.
6. *Ferro M., Salvis D., Brugière S.* Proteomics of the chloroplast envelope membranes from Arabidopsis thaliana. *Mol. Cell. Proteom*. 2003, V. 2., P. 325–345. doi: 10.1074/mcp.M300030-MCP200.
7. *Hall J. L.* Cellular Mechanisms for Heavy Metal Detoxification and Tolerance. *J. Exp. Bot*. 2002, V. 53, P. 1–11.
8. *Molas J.* Changes in morphological and anatomical structure of cabbage (*Brassica oleracea* L.) outer leaves and in ultrastructure of their chloroplasts caused by an *in vitro* excess of nickel. *Photosynthetica*. 1997, 34 (4), 513–522.
9. *Molas J.* Changes of chloroplast ultrastructure and total chlorophyll concentration in cabbage leaves caused by excess of organic Ni (II) complex. *Environ. Exp. Bot*. 2002, V. 47, P. 115–126.
10. *Vassilev A., Lidon F., Scotti P., Da Graca M., Iordanov I.* Cadmiuminduced changes in chloroplast lipids and photosystem activities in barley plants. *Biol. Plant*. 2004, 48 (1), 153–156.
11. *Krupa Z., Baszyński T.* Some aspects of heavy metals toxicity towards photosynthetic apparatus – direct and indirect effects on light and dark reactions. *Acta Physiol. Plant*. 1995, V. 17, P. 177–190.
12. *Pätsikkä E., Aro E.-M., Tyystjärvi E.* Mechanism of cupper-enhanced photoinhibition in thylakoid membranes. *Physiol. Plant*. 2001, V. 113, P. 142–150.
13. *Vodka M. V., Polishchuk A. V., Belyavskaya N. A., Zolotareva E. K.* Effects of heavy metals, carbonic anhydrase inhibitors, on photosynthetic apparatus of pea leaf chloroplasts. *Biul. Kharkiv Nats. Ahrar. Univ. Ser. Biol*. 2013, 30 (1), 46–55. (In Russian).
14. *Vodka M. V., Polishchuk O. V., Bilyavs'ka N. O., Zolotareva E. K.* Response of spinach photosynthetic apparatus to the action of heavy metals, carbonic anhydrase inhibitors. *Reports of the NAS of Ukraine*. 2013, V. 10, P. 152–158. (In Ukrainian).
15. *Titov A. F., Talanova V. V., Kaznina N. M., Laidinen G. F.* Resistance of plants to heavy metals. *Petrozavodsk: Karelian Research Centre of the RAS*. 2007, P. 172. (In Russian).

## СТРУКТУРНО-ФУНКЦІОНАЛЬНІ ЗМІНИ ХЛОРОПЛАСТІВ ЯК БІОМАРКЕРИ ЗАБРУДНЕННЯ ВАЖКИМИ МЕТАЛАМИ

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Метою роботи було підтвердження гіпотези щодо можливості використання структурно-функціональних змін хлоропластів вищих рослин як біомаркерів для оцінки забруднення важкими металами.

Ультраструктурні зміни хлоропластів *Pisum sativum* L. реєстрували методом трансмісійної електронної мікроскопії. У роботі досліджено реакцію ультраструктури хлоропластів на підвищений вміст міді (250 μmM) і цинку (400 μmM). Отримано дані про зміни у структурі хлоропластів, зокрема неоднорідність упаковки гран тилакоїдів, збільшення міжтилакоїдних проміжків і товщини гран тилакоїдів хлоропластів порівняно з контролем. Результати досліджень стосовно структурно-функціональних змін хлоропластів можна застосовувати як маркери за ранньої діагностики впливу абіотичних стресів, зокрема важких металів, а також у біотехнологічних дослідженнях при створенні нових стійких до стресів високотехнологічних сортів сільськогосподарських культур.

***Ключові слова:*** *Pisum sativum* L., зміни хлоропластів, забруднення важкими металами, біомаркери.

## СТРУКТУРНО-ФУНКЦИОНАЛЬНЫЕ ИЗМЕНЕНИЯ ХЛОРОПЛАСТОВ КАК БИОМАРКЕРЫ ЗАГРЯЗНЕНИЯ ТЯЖЕЛЫМИ МЕТАЛЛАМИ

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Целью работы было подтверждение гипотезы о возможности использования структурно-функциональных изменений хлоропластов высших растений в качестве биомаркеров для оценки загрязнения тяжелыми металлами.

Ультраструктурные изменения хлоропластов *Pisum sativum* L. регистрировали методом трансмиссионной электронной микроскопии. В работе исследована реакция ультраструктуры хлоропластов на повышенное содержание меди (250 μmM) и цинка (400 μmM). Получены данные об изменениях в структуре хлоропластов, в частности неоднородность упаковки гран тилакоидов, увеличение межтилакоидных промежутков и толщины гран тилакоидов хлоропластов по сравнению с контролем. Результаты исследований о структурно-функциональных изменениях хлоропластов можно применять в качестве маркеров при ранней диагностике влияния абиотических стрессов, в частности тяжелых металлов, а также в биотехнологических исследованиях при создании новых устойчивых к стрессам высокотехнологичных сортов сельскохозяйственных культур.

***Ключевые слова:*** *Pisum sativum* L., изменения хлоропластов, загрязнение тяжелыми металлами, биомаркеры.