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CHLOROPLASTS ULTRASTRUCTURAL CHANGES AS BIOMARKERS OF ACID RAIN AND HEAVY METALS POLLUTION

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The aim of the work was to confirm the possibility of structural changes of *Spinacea olearacea* L. chloroplasts usage as biomarkers for assessing of environmental pollution by acid rain and heavy metals. Chloroplasts ultrastructural changes were recorded by transmission electron microscopy. Data on changes in the structure of chloroplasts under the influence of these factors are obtained, in particular the heterogeneity of thylakoid grana packing, the membranes thickness, the starch grains presence, and the lumen space increase as compared with the control. These structural changes can be applied as markers of abiotic stresses influence, notably acid rain and heavy metals, and for the creation of new sustainable high-tech varieties of agricultural crops.

Key words: *Spinacea olearacea* L., imitated acid rains, heavy metals, chloroplast structure, biomarkers.

Recently, among the most dangerous environmental impacts, the influence of acid rain and heavy metals is named, because they cause not only numerous diseases in humans and animals, but also significant changes in the representatives of plant world. Increased water acidity contributes to higher solubility of hazardous metals such as cadmium, mercury and lead from bottom sediments and soils. Soil acidification as a result of atmospheric pollution leads to changes in species diversity of natural ecosystems.

A complex mixture of pollutants, including acid rain and heavy metals leads totally to the forests degradation. Acid rain is especially characteristic for countries with highly developed urbanization, such as US, Japan and China. In our country, more and more acid rain falls in the industrial regions of the southeast. The impact of acid rain is realized either directly (primarily on the leaves) or indirectly (through the soil on the root system) [1]. All kinds of meteorological precipitation (rain, snow, hail, snowy rain, fog) in which pH is decreased due to the air pollution with acidic oxides are called acid rains. In normal rainwater pH indicator is 5.6, while rain with $pH < 5$ is considered acid rain [2].

Acid rain is formed by reaction between water and substances such as sulfur dioxide (SO_2) and nitrogen oxides (NO_x). Pollutants are emitted into atmosphere by motor transport, iron and steel companies and thermal power plants, as well as at coal and wood burning. Reacting with atmosphere water, they turn to acid solutions — sulfuric, sulfurous, nitrous and nitric. Then with snow or rain, they fall on the ground.

It should be noted that anion SO_4^{2-} most (60%) contributes to the acidification of precipitation, since the fall-out of sulfur with precipitation is 4 times higher than the fall-out of nitrogen [3]. At this, anions SO_4^{2-} cause inhibitory effect on various processes in photosynthetic cells.

Sulfur dioxide penetrate into the plant, where it is involved in various oxidative processes. They proceed with the free radicals formed from sulfur dioxide as a result of chemical reactions, and oxidize membrane unsaturated fatty acids, thus altering their permeability, which can further negatively influence the different processes (respiration, photosynthesis, etc.). In addition, there is stomatal closing cells damage, leaving them open, dramatically increasing transpiration and, consequently, increasing the content of toxicants, which in turn, increases cell damage [4]. At pH 2.0 appreciable plant damages become visible, the dry weight, photosynthetic CO_2 fixation and photochemical activity decrease [5, 6].

Competitive inhibitor of diphosphate carboxylase is sulfur dioxide, which prevents CO_2 fixation in photosynthesis. It is known that acid rain also lead to a decrease in chlorophyll and carotenoids [7]. Hogan and Taylor [8] found that acid rain of the pH 3.5 does not cause the primary stress, but of the pH 2.3–3.0 the chlorophyll content increase and the photosynthetic rate reduction were observed [9].

To date, experiments on plants studying the effects of acid rain on the ultrastructure and biochemical parameters are limited. Thus, several authors found the ultrastructure of cells violations in harsh acidification [10–12]. Major ultrastructural changes under the influence of acid rain were observed in mesophillous layers, namely, in chloroplasts and mitochondria [10], because it is known that the change in chloroplast structural state is the primary universal response of the photosynthetic apparatus in response to the change in any environmental condition [13], including the lengthy blackout [14], salt excess in the soil [15], aerobiosis development under the influence of physical and chemical agents [16].

In our previous work, the possibility of pea chloroplasts structural and functional characteristics usage as biomarkers of contamination with heavy metals has been shown [17]. The data have shown the presence of changes in the structure of chloroplasts, in particular, we have observed heterogeneity of thylakoid grana packing, increase the lumen space and thylakoid membranes thickness of chloroplast grana compared to control. In order to more detailed and reasonable determination and prediction of adverse effects of environmental factors such as acid rain and thereof mixtures with heavy metals in various plant processes (photosynthesis et al.), it was expedient to verify and confirm the changes in chloroplasts of spinach leaves ultrastructure as biomarkers of environment pollution.

Materials and Methods

In experiments, the spinach *Spinacea olearacea* L. leaves (4–6-week old) were used which were grown under controlled conditions and under the influence of acid rain and heavy metals.

Spinach plantlets once sprayed for 3 min with 1 liter of equimolar (0.2 mM) mixture of $NaNO_3$ and Na_2SO_4 solutions, pH 5.6 (control) or 2.5 (acid variant). To determine the action of heavy metals, spinach leaves were treated with 80 μM Cu^{2+} ($CuSO_4$) or 200 μM Zn^{2+} ($ZnSO_4$).

Then the plants were kept 2 days more at 22 °C and luminance of 150 μmol quanta/(m^2·s), after that the samples of spinach leaves were investigated by transmission electron microscopy (TEM).

From the middle part of the half of spinach plantlet leaves the fragments of 2×2 mm were cut. Samples fixation was performed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 4 hours at 4 °C. After washing in the same buffer (2×20 min), material postfixation was performed with 1% OsO_4 solution in 0.1 M cacodylate buffer overnight at 4 °C. Samples dehydration was conducted in ethanol of increasing concentration and acetone. After that, the material was soaked in a mixture of epoxy resins and acetone, poured in epone araldyte resin using conventional method. Then the blocks were transferred to a thermostat for the polymerization at 60 °C for 3 days.

Ultrathin cells sections of 80–100 nm thickness were made using ultramicrotome LKB-V (LKB, Sweden). Sections were contrasted with a mixture of uranyl acetate and potassium permanganate (1: 1) for 15 min in the darkness. Ultrathin sections were examined and photographed with a transmission electron microscope JEM-1300 (JEOL, Japan) for 80 min.

For morphometric analysis, the photos of chloroplasts and their fragments were scanned with Epson Perfection 3200 Photo scanner. Photos were prepared using Adobe Photoshop 7.0 and Corel Photo-Paint 11 computer programs. For each variant of test and control, 30–39 photos with ×10 000, 15 000 or 100 000 increasing were analyzed.

Determination of grana elements size in chloroplast images was carried out by ImageTool 3.0 (UTHSCSA, USA) computer program. Processing of research results was carried out using applied software package Microsoft Excel 7.0, BIO8, Statistica 6.0. The values of average and standard quadratic deviation were calculated, which, including data from all three repetitions of experiment, did not exceed 5%. Experimental data are presented as the arithmetic mean (M) with a standard deviation (m), defined with all repetitions. Experiments were repeated three times. The reliability of the difference between the mean values of experimental and control variants were evaluated using Student *t*-test [18]. The difference was considered statistically significant for $P \leq 0.05$.

Results and Discussion

Fig. 1 shows the cut of spinach leaf chloroplast of control variant. Chloroplast has lenticular form. The internal membrane system (stroma grana and thylakoids) form several rows, almost parallel to the long axis of chloroplasts, among which there are single plastoglobules (*a*). Low grana contain from 10 to 20 densely packed thylakoids (*b*). Stroma has a moderate electron density.

The impact of acid rain on the chloroplasts of spinach leaves structure is shown in Fig. 2. The characteristic feature of this effect is the loss of regularity of internal membrane system layout in strict rows (*a*), as it is observed in the chloroplasts of the control samples. The dense packing of the grana is lost due to significant and irregular increase of lumen area. Obviously, this is due to significant swelling of all membrane elements leading to their spatial organization disturbance, undulation appearance, particularly in stromal thylakoids (*b*). The massive membrane conglomerates are formed, the number of plastoglobules increases.

It is shown that under conditions simulating acid rain, the membrane thickness of thylakoid grana and the width of the lumens space only have tend to increase compared with the figures in the control samples.

Under the influence of simulated acid rain with the addition of copper ions a part of chloroplasts changes lenticular form on amoeba form (Fig. 3, *a*). Weak undulation of thylakoid stroma is found; grana intactness remains, however, grana show a swollen configuration (Fig. 3, *b*).

At the joint action of simulating acid rain and Cu^{2+} ions, the membrane thickness of thylakoid grana and the width of lumens space have tend to increase compared with the control.

Fig. 4 shows the effect of acid rain with the addition of zinc ions. Lenticular chloroplasts form retains, but most of its cut is occupied by large starch grains, which put pressure on the membrane elements, pushing them to the periphery of chloroplast (*a*). Meanwhile the grana and stroma thylakoids show some swelling and waviness (*b*). Rare single plastoglobules are found.

It is found that under conditions simulating acid rain mixed with Zn^{2+} ions, the width of lumen space and membrane thickness of grana thylakoids increase compared with the figures in the control samples.

Some effects of acid rain on chloroplasts were found in the leaves of plants *Lycopersicon esculentum* Mill., in which chloroplasts rounding, thylakoid swelling, conglomerates formation, thylakoids wavy configuration and appearance of several starch grains were also observed [10]. Similar changes were recorded in the chloroplasts in the leaves of *Phaseolus vulgaris* after spraying with a solution of substances that are the part of acid rain [11]. Thylakoids waviness and swelling, and the thickness of chloroplast grana increasing were registered in moss *Bryoria fuscessens*, which was treated with acid rain in combination with copper ions [12].

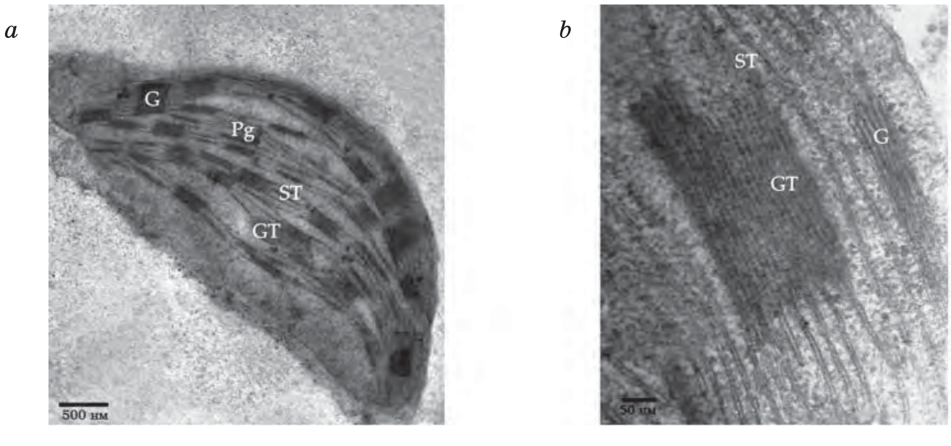

Fig. 1. **Chloroplasts (*a*) and grana (*b*) ultrastructure of spinach leaves: control**
Hereinafter: G — granum; Pg — plastoglobule;
GT — granum thylakoids; ST — stroma thylakoids

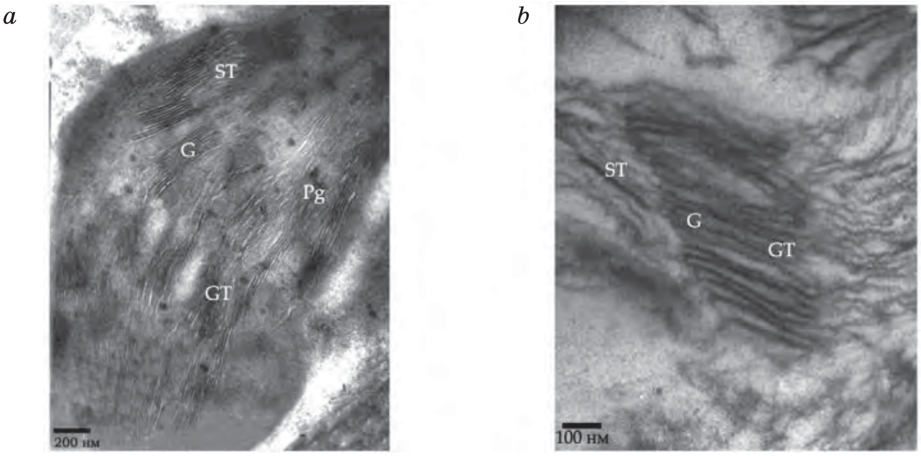

Fig. 2. **Chloroplasts (*a*) and grana (*b*) ultrastructure of spinach leaves:**
acid rain

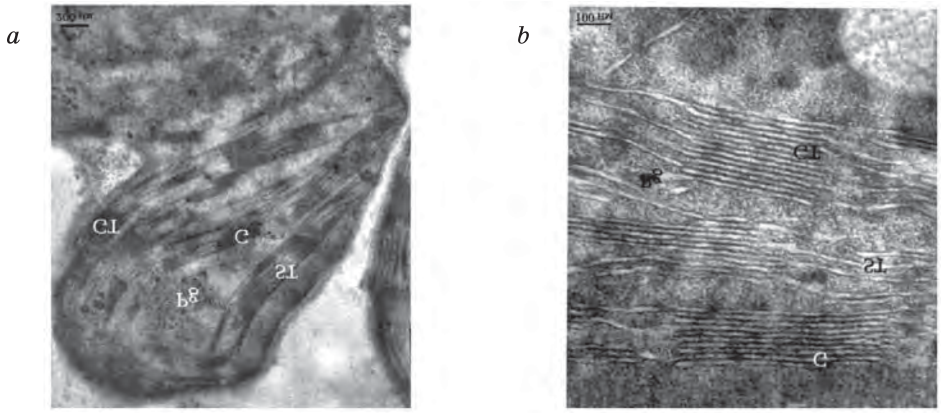

Fig. 3. **Chloroplasts (*a*) and grana (*b*) ultrastructure of spinach leaves:**
a mixture of acid rain and Cu^{2+} ions

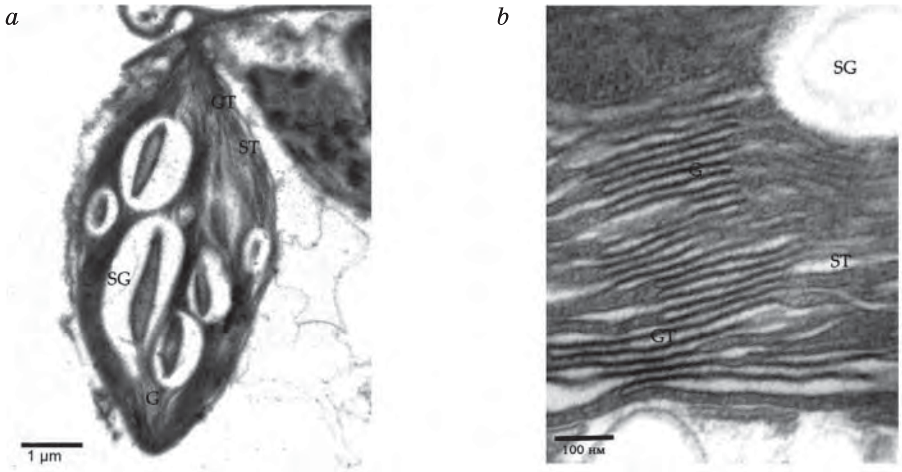

Fig. 4. **Chloroplasts (*a*) and grana (*b*) ultrastructure of spinach leaves:**
a mixture of acid rain and Zn^{2+} ions; SG — starch grain

As shown in Fig. 5, under the influence of Cu^{2+} ions the form of chloroplasts becomes more rounded. It should be noted the decrease of inner membrane volume, stroma thylakoids tortuosity and some enlightenment of chloroplasts stroma compared with control samples. Numerous rounded plastoglobules of larger size as compared to the control chloroplasts, often form groups comprised 3 to 10 inclusions that may be closely adjacent to each other (*a*). Under the influence of Cu^{2+} ions the thylakoid membrane thickness is slightly increased, while the width of the lumen space is slightly changed.

Fig. 6 shows the general structure of chloroplasts that does not undergo significant changes; however, plastoglobules population undergoes rearrangements (their size and number are increased). It should also be noted that plastoglobules morphology is atypical: most of them have well-defined outer boundary, in the middle of inclusions only a few islands of electron-dense material are observed, outside of which you can see thylakoid profiles (*b*). There is an asymmetry of certain grana. The density of the stroma is not differing from that of the control. Under the influence of Zn^{2+} ions, an increase in membrane thickness of grana thylakoids and expansion of lumen space in chloroplasts occur.

The results of these studies suggest that the combined effect of acid rain and copper ions reduces the negative impact of certain factors on the parameters of the photosynthetic

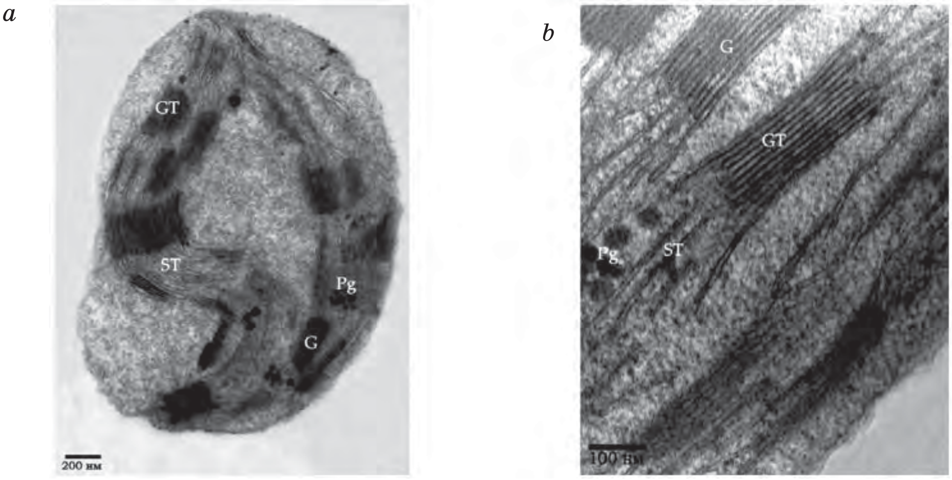

Fig. 5. **Chloroplasts (*a*) and grana (*b*) ultrastructure of spinach leaves:**
Cu^{2+} ions

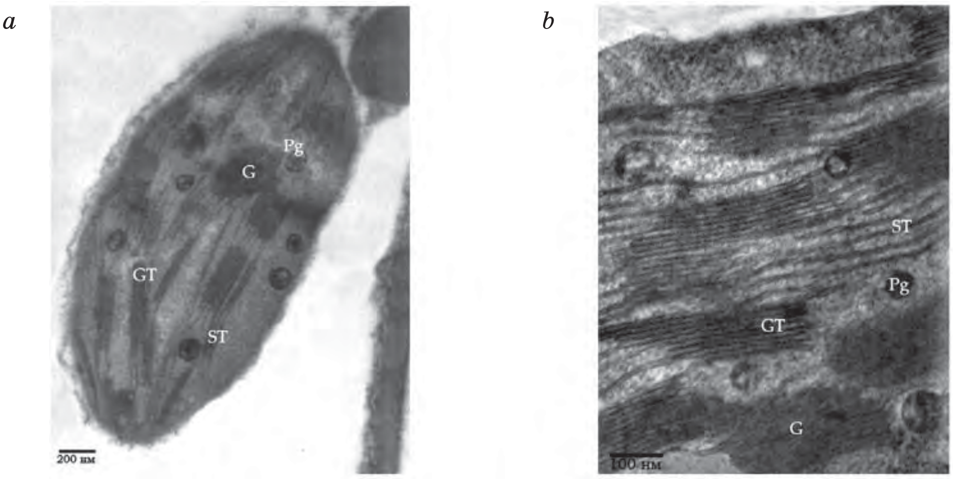

Fig. 6. **Chloroplasts (*a*) and grana (*b*) ultrastructure of spinach leaves:**
Zn^{2+} ions; *M* — mitochondrion

apparatus, possibly by antioxidant status increasing — activity of superoxide dismutase and catalase strengthening, either/or growth of thiols level. This antagonism may be associated with the reduction of heavy metal entering into the cell.

A significant effect is observed under the influence of acid rain with the addition of zinc ions. The emergence of large starch grains is common feature of influence on chloroplasts. It is likely that the increased content of starch in the chloroplast stroma of test plants is caused by its degradation delay, which is consistent with data from other authors on the disappearance or significant decrease in starch content against the backdrop of plastids ultrastructural organization restructuring during light phase of photoperiod [18, 19]. Reducing the degradation of transient starch observed in our case can be explained also by violation of the outflow of starch hydrolysis products from plastid caused by the reduction in metabolism intensity under the influence of acid rain and zinc ions. It is assumed that the starch degradation products retain in the disengaged state after its disintegration. At this, round the residues of starch grains the area of low electron density are formed, which are well manifested in electron micrographs (Fig. 5). It is obviously that the excess amount of photosynthesis products is transformed into the starch. Starch storage along with other ultrastructural changes occurs likely as a result of imbalance between photosynthesis and its products export [20]. A large amount of starch that accumulates in starch grains, destroys the intact chloroplasts structure from plants grown in the presence of Zn^{2+} ions.

Summarizing the findings on the impact of acid rain on experimental plants, one could argue that they cause significant ultrastructural changes in chloroplasts.

Tortuosity of stroma thylakoids structure observed in spinach chloroplasts under the influence of copper and zinc ions was found in the chloroplasts of *Elsholtzia splendens* [20, 21] and *Salix purpurea* [22] leaves treated with copper ions. In the leaves of *Zea mays* plantlets, asymmetry of chloroplast grana is revealed after prolonged treatment with zinc ions [23], as it is in part of spinach leaves grana in our experiments. However, it should be noted that in our samples from spinach leaves there are no cases of chloroplast stroma compressing, like in corn leaves, which apparently can be explained by the different time of plantlets processing with zinc ions.

The most common reaction to the effects of various factors, including heavy metals, is the increase of the number and size of plastoglobules, as it is observed in our samples under the action of copper ions. The same phenomenon is found in the chloroplasts of cells in suspension culture *Glycine max* [24], leaves of *Pisum sativum [25], Oreganum vulgare [26], Elsholtzia splendens [21], Salix purpurea* and *Phragmitis australis* [22] after similar treatment.

A special feature of leaves processing with zinc ions is plastoglobules content fragmentation and partial it washout leaving intact the outer layer. As it is known, plastoglobules are surrounded outside by polar lipid monolayer, which communicates with an external lipid leaf of thylakoids [27, 28]. Plastoglobules surface is studded with proteins [28]. Among them, there are fibrillins (plastoglobulins), which are considered structural, apparently involved in lipids metabolism. The middle of plastoglobules consists of neutral lipids, tocopherol, phylloquinone, carotenoids, phytic esters of fatty acids, triacylglycerols, and others [29]. The amount, size and lipid content vary depending on ambient conditions and stage of plant development [30]. Heavy metals can trigger the emergence of oxidative stress [31], among its displays the lipid oxidation is particularly dangerous because of so-called "chain reaction" resulting in formation of free radicals. Lipid peroxidation (LPO) is regarded as one of the main signs of oxidative stress [32]. It is possible that this oxidative stress is the cause of partial plastoglobules degradation observed in spinach chloroplasts.

Thus, the treatment of spinach leaves with zinc and copper ions caused the modifications in chloroplasts structure, which can be classified as moderate impact. Obviously, such a moderate reaction of ultrastructure of plastids from the intact spinach plantlets leaves treated with heavy metal ions can be explained as by short-term exposure to their solutions, and by low flow of ions into the cells of leaves due to their active binding to the components of cell coats, as this was observed in *Elsholtzia splendens* [33], *Paulownia tomentosa* [34] along with their compartmentalization in vesicles or vacuoles [34, 35]. Plants have developed a complex network of homeostatic mechanisms to modulate internal concentration of heavy metals in the cytosol and maintain their toxicity below a certain level [36].

Thus, studies have found the moderate effect of heavy metals on the structure of chloroplasts in leaves of spinach plantlets.

In our opinion, the results of research on the example of spinach and peas [17] for chloroplasts structural changes can be used as markers to identify the influence of abiotic stresses, including acid rain and heavy metals.

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УЛЬТРАСТРУКТУРНІ ЗМІНИ ХЛОРОПЛАСТІВ ЯК БІОМАРКЕРИ ЗАБРУДНЕННЯ КИСЛОТНИМИ ДОЩАМИ ТА ВАЖКИМИ МЕТАЛАМИ

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Метою роботи було підтвердити можливість використання структурних змін хлоропластів *Spinacea olearacea* L. як біомаркерів для оцінки забруднення навколишнього середовища кислотними дощами і важкими металами. Ультраструктурні зміни хлоропластів реєстрували методом трансмісійної електронної мікроскопії. Отримано дані про зміни у структурі хлоропластів під впливом зазначених чинників, зокрема неоднорідність упаковки гран тилакоїдів, товщини мембран, наявність крохмальних зерен, збільшення люменального простору порівняно з контролем. Ці структурні зміни можна застосовувати як маркери для вивчення ефектів абіотичних стресів, зокрема кислотних дощів та важких металів, а також для створення нових, стійких високотехнологічних сортів сільськогосподарських культур.

Ключові слова: *Spinacea olearacea* L., імітовані кислотні дощі, важкі метали, структура хлоропластів, біомаркери.

УЛЬТРАСТРУКТУРНЫЕ ИЗМЕНЕНИЯ ХЛОРОПЛАСТОВ КАК БИОМАРКЕРЫ ЗАГРЯЗНЕНИЯ КИСЛОТНЫМИ ДОЖДЯМИ И ТЯЖЕЛЫМИ МЕТАЛЛАМИ

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Целью работы было подтвердить возможность использования структурных изменений хлоропластов *Spinacea olearacea* L. в качестве биомаркеров оценки загрязнения окружающей среды кислотными вождями и тяжелыми металлами. Ультраструктурные изменения хлоропластов регистрировали методом трансмиссионной электронной микроскопии. Получены данные об изменениях в структуре хлоропластов под влиянием указанных факторов, в частности неоднородность упаковки гран тилакоидов, толщины мембран, наличие крахмальных зерен, увеличение люменального пространства по сравнению с контролем. Эти структурные изменения можно применять в качестве маркеров для изучения эффектов абиотических стрессов, в частности, кислотных дождей и тяжелых металлов, а также для создания новых устойчивых высокотехнологичных сортов сельскохозяйственных культур.

Ключевые слова: *Spinacea olearacea* L., имитированные кислотные дожди, тяжелые металлы, структура хлоропластов, биомаркеры.