It was shown that clinostating conditions induce autophagy without increasing of programmed cell death (PCD) index in the epidermal cells of the root apex of *A. thaliana* seedlings. After the phase of activation of autophagy, its regulatory weakening occurs, which probably indicates adaptive changes to the conditions of clinostating. The induction of autophagy correlates with an increase in the expression levels of *atg8* genes, some of which (*atg8e* and *atg8i*) may be involved in the implementation of autophagy under the simulated microgravity conditions. The transcriptional activity of cytoskeleton genes involved in the implementation of stress-induced autophagy, in particular *α*- and *β*-tubulin genes, was analyzed. Joint expression of *α*- and *β*-tubulin genes and *atg8* under the simulated microgravity conditions was revealed. These results illustrate the role of the cytoskeleton in the development of microgravity-induced autophagy and make it possible to identify genes specific to this type of stress.

The induction of autophagy and PCD was studied under the action of gamma irradiation as a concomitant factor of space flights, as well as under the combined action of acute irradiation and clinostating. Gamma irradiation in doses equivalent to those in the spacecraft cabin (1 - 6 Gy) induced dose-dependent changes in the topology and cytogenetic state of the root apical meristem, as well as slightly inhibited of the early plant development. In the meristem, heterogeneity increased, PCD indexes, mainly proliferative death and autophagy, increased. With the combined action of gamma irradiation (2 Gy) and clinostating, the density of autophagosomes in the epidermal cell root apices of 6-day-old seedlings increased (24 hours after irradiation), and after 4 days it decreased, compared to the non-irradiated control.
Treatment of seeds of A. thaliana with a NO donor had a stimulating effect on plant development, increased the content of endogenous NO in root tissues and the resistance of plants to clinostating. During clinostating the concentration of NO decreased, possibly due to the contribution of NO to the generation of ROS. The negative effect of NO scavenger on seedling growth was enhanced by clinostating, including increased accumulation of autophagosomes in epidermal cells. These data indicate that endogenous NO content is an important component of intracellular signaling mechanisms involved in the response of plant cells to simulated microgravity, including autophagy induction mechanisms. The obtained data deepen the understanding of the molecular mechanisms of the development of stress-induced autophagy, in particular the involvement of different isotypes of ATG8 proteins and their interaction with α- and β-tubulins, as well as other molecular components involved in the induction of autophagy, and will be the basis for the development of approaches to increase stress resistance and adaptation of plants to the conditions of long-term space flights.

**Keywords**: autophagy, atg8 genes, genes of α- and β-tubulins, nitric oxide, adaptation, clinostating, gamma irradiation, Arabidopsis thaliana.

1. **INTRODUCTION**

Space experiments and experiments with simulated microgravity have shown that the reorganization of plant cell structure and metabolism depend on the taxonomic position and physiological state of the research objects, the growth phase, and the duration of exposure to real or simulated microgravity [11]. Under the conditions of microgravity, ultrastructural reorganization of organelles and changes in the calcium balance occur, the physicochemical properties of the plasma membrane change, and the activity of enzymes increases. These events cause acceleration of cell growth and differentiation and premature aging of plants. Complex signaling, which is involved in the response to stress, results in changes in gene expression and lateral distribution of auxin at the sites of reception [10]. Some responses can be seen as adaptive to microgravity conditions. Under the conditions of microgravity, the expression of genes coding for proteins responsible for cell wall metabolism, hormonal status, system polarity, cytoskeleton, oxidative stress, lipid metabolism, cell division, and PCD development is significantly modified [4, 6, 11, 19]. One of the most interesting issues related to microgravity is the induction of autophagy, which is the organism’s adaptive response to stress. We have recently shown that the development of autophagy in plant cells can be initiated by various abiotic stresses – starvation, osmotic and salt stress, UV-B irradiation [15, 19, 20]. Although it is considered an important mechanism for maintaining cellular homeostasis, including different stresses, the physiological role and regulation of autophagy in plants remain insufficiently studied [14—16, 19, 20, 28].

Recent studies have established a functional relationship between α-tubulin acetylation and the development of stress-induced autophagy in plants [18, 21]. Expression patterns of α-tubulin genes, ATG8 protein genes and genes of enzymes involved in α-tubulin acetylation, as well as kinesin genes (motor proteins of microtubules), which may be involved in mediating autophagy processes involving microtubules, have been characterized [16, 18]. Inhibition of autophagy by the cysteine protease inhibitor E-64 under stress conditions (starvation, osmotic and salt stress, etc.) led to inhibition of plant growth, which confirms the adaptive role of autophagy [21]. However, the influence of such a factor as microgravity on the development of autophagy and the importance of this process for the adaptation of plants to weightlessness conditions remain unexplored. The study of autophagy and PCD becomes especially relevant with the development of ways to increase stress resistance and adaptation of plants to microgravity conditions. An example of works in this direction is the modification and correction of signaling pathways, in particular, with the participation of nitric oxide (NO), which increase the stress resistance of plants [4, 7, 9]. In particular, NO increases resistance to such stresses as drought, salinity, UV-B irradiation, exposure to heavy metals and low temperatures [4, 17, 23]. However, the role of NO in the mechanisms of adaptation to microgravity, including the possible induction autophagy, has not yet been studied.

The aim of the work was to study the induction of autophagy as an adaptive mechanism to simulated microgravity conditions, including the analysis of the transcriptional profiles of the atg8, tubulin genes and the role of NO in the mechanisms of adaptation to simulated microgravity using NO donors and scavengers. Detailed information about used research methods can be found in our works [18—20, 24, 26].
2. RESULTS OF RESEARCH AND DISCUSSION

2.1. The effect of clinostating on the growth of seedlings, the appearance of autophagosomes and the dynamics of autophagy in the cells of the apex of the main root of A. thaliana. Although experimental data about the possible influence of microgravity on autophagy processes in plant cells were practically absent until recently, we found that under clinostating, plants experience a certain lack of vital resources due to the weakening of the export of assimilates to the stem and root apices, which, with time, is overcome due to compensatory regulatory relationships [13]. It was logical to assume that as compensatory mechanisms of adaptation to microgravity conditions, plants can activate autophagy mechanisms. Therefore, the goal of the first stage of our research was to identify the first signs of seedling growth inhibition and cytological analysis of autophagy in cells of the main roots under clinostating conditions. The main results of these studies are presented in our previous publications [24, 26, 28].

Clinostating increased the degree of size variation and changed the spatial orientation of seedlings [13, 24, 28]. The difference between experimental and control plants was most pronounced in 5-6-day-old seedlings, as a result of inhibition of growth of clinostated seedlings; later this difference leveled out. After cell staining with propidium iodide (PI), it was shown that root cells of 6-10-day-old seedlings under clinostating conditions were characterized by high survival rates. The PCD index was within 5 %, which is the physiological norm for A. thaliana, and indicates that the development of autophagy did not lead to the activation of apoptosis.

Analysis of root apex tissues using two autophagosome markers — monodansyl cadaverine and LysoTracker™ Red — showed that the density of autophagosomes in the root apices of clinostated plants was significantly higher than in the control [24, 25, 28]. The dynamic of the formation of autophagosomes in the root tissues of control and experimental plants was different. Under the conditions of clinostating, in 6-day-old seedlings in the epidermal cells of the meristematic zone, later, and in the transition zone, activation of autophagy was found (Fig. 1). On the 9th and 12th day of the study, the number of autophagosomes decreased. In the zone of root extension, the number of autophagosomes reached its maximum on the 10th day of clinostating. Instead, in the cells of the root cap on the 10th day of clinostating, the number of autophagosomes rapidly decreased, which is obviously related to the renewal cycle of these cells. In control plants, the density of autophagosomes in epidermal cells was inferior to that of clinostated plants and reached a maximum later. Weakening of autophagy activity after reaching a maximum may indicate the self-regulation of its nature — the creation of a certain regulatory optimum of the vital resources level [24, 26, 28].

2.2. The influence of clinostating on the transcriptional activity of atg8 genes. The goal of the next part of the research was to identify the relationship between changes in autophagy development and the transcriptional activity of atg8 genes involved in the formation of autophagosomes under simulated microgravity. The obtained results are presented in the publications [24, 26].

Using molecular genetic analysis of the expression profiles of 9 atg8 genes it was found that the changes in the expression of these genes depend on the duration of clinostating. In particular, the expression levels of the atg8e, atg8f and atg8i genes increased on the 6th, 9th, and 12th days of clinostating (Table 1). Changes in the expression levels of atg8i gene during clinostating were the highest compared to other atg8 genes. The expression of atg8a, atg8b, atg8c, and atg8d genes increased on the 6th day of clinostating, and dramatically decreased on the 9th and, especially on the 12th day [24, 26]. The most sensitive to clinostating was the atg8h gene. Its expression changed dramatically during clinostating (Table 1).

Comparison of these data with gene expression levels of atg8 genes under such stress as starvation, salt and osmotic stress, and UV-B irradiation [19, 20, 28] indicates that gene expression levels of atg8e increase 1.5—2 times under the action all these stress factors, except atg8f — only UV-B did not affect its expression. For the atg8i gene higher expression levels were noted only under starvation and clinostating. For the atg8g gene, no increase in expression levels was detected during clinostating, while its expression levels increased under salt stress and UV-B [19, 20, 26]. These data may indicate that atg8g and atg8h genes are not directly involved in the implement-
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Considering the fact that the ATG8 protein is a structural unit of autophagosomes and is directly involved in the development of stress-induced autophagy the obtained results also indicate that a part of the \textit{atg8} (\textit{atg8e} and \textit{atg8i}) genes may be involved in the full implementation of autophagy under simulated microgravity. This assumption is supported by the increase in the expression level of these genes under starvation [20].

2.3. The influence of simulated microgravity on the transcriptional activity of \(\alpha\)- and \(\beta\)-tubulin genes. The transcriptional activity of genes involved in the implementation of stress-induced autophagy, in particular the \(\alpha\)- and \(\beta\)-tubulin genes of \textit{A. thaliana} under microgravity conditions was present in [25]. As a result of the analysis of the expression of 6 \(\alpha\)- and 9 \(\beta\)-tubulin genes, an increase in the expression levels of \textit{tua2}, \textit{tua3}, \textit{tua4}, \textit{tua6} genes and a decrease in expression of \textit{tua4}, \textit{tua5} genes on the 6\textsuperscript{th} and 9\textsuperscript{th} days of cultivation were found (Table 1). On the 12\textsuperscript{th} day of plant growing under clinostating conditions, none of the six studied genes showed an increase in the level of expression (Table 1) [25]. The \textit{tua1} gene is probably not involved in autophagy, as its expression level was consistently low during long-term cultivation [20].

As the result of the comparative analysis of the expression profiles of all studied genes, joint upregulation of the \(\alpha\)- and \(\beta\)-tubulin genes and \textit{atg8} genes under the influence of microgravity was found (Table 1). These results illustrate the role of the microtu-
bules in the development of stress-induced autophagy, and provide an opportunity to identify genes specific to stress induced by simulated microgravity. In particular, it was established that certain *tua*, *tub* and *atg8* genes can be considered as key elements of plant adaptation to prolonged exposure to microgravity [25]. Thus, data on the expression of plant tubulin genes under clinostating were obtained for the first time, that provides a basis for the development of approaches to increase the adaptation of plants during their growing under the influence of such stress.

2.4. Induction of radiation damage in the root meristem after gamma irradiation as a concomitant factor of space flights. The induction of radiation damage, autophagy, and PCD was studied under the action of gamma irradiation, as well as under the combined action of gamma irradiation and clinostating. The effect of gamma irradiation in doses equivalent to those in the spacecraft cabin (from 1 to 6 Gy) was studied on *Pisum sativum* seedlings, which is an appropriate model for cytogenetic studies. The development of plants after irradiation was inhibited in a dose-dependent manner. Gamma irradiation causes dose-dependent changes in the topology and cytogenetic state of the root apical meristems (Fig. 2). The first signs of increased cell heterogeneity were visible at 2 Gy irradiation. When the radiation dose increased to 4—6 Gy, the heterogeneity of cell populations in the apical meristem increased, the number of cells with chromosomal aberrations and induced PCD were increased (Fig. 2). The spectrum of chromosomal aberrations was dominated by single and double bridges and fragments, less often — by multiaberrant cells and micronuclei (Fig. 2, g–j).

The main form of PCD was the proliferative death of multiaberrant cells. The dose dependences of the induction of chromosomal aberrations in the irradiation range up to 4 Gy were characterized by linearity, and in the range of 4—6 Gy with an index of 30 % of aberrant anaphases, they reached a plateau due to the cytostatic effect and PCD. The combined effect of gamma irradiation (2 Gy) and clinostating did not significantly affect the growth and morphogenesis of the main roots of *A. thaliana*. In the seedlings of the AtGFP-MAP4 line under these conditions reorientation of cortical microtubules in the distal part of the roots was observed. With the combined effect of clinostating and irradiation the number of autophagosomes in the root meristematic cells of 7-day-old seedlings increased significantly after 24 h of exposure, and later (in 4 days) decreased, compared to the non-irradiated control.

2.5. Induction by clinostating of heat shock proteins and cross-adaptation. In addition to starvation, autophagy can be induced by oxidative stress and/or the accumulation of partially denatured proteins and their aggregates in the cytoplasm (chaperone type of autophagy found in mammals). The subsequent type of autophagy development may be mediated by chaperones of the HSP70 family. Spaceflight factors, including gravity shift have recently been shown to enhance the expression of HSP proteins in plant cells. So, together with colleagues [12], it was established that clinostating stimulates the response to heat shock (expressed *AtHSP70* and *AtHSP90-I*) in *A. thaliana* seedlings, which was confirmed both at the level of transcription and translation [12]. The obtained results indicate that seedlings after long-term clinostating are able to withstand the influence...
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2.6. Development of ways to increase plant stress resistance to microgravity. The role of nitric oxide in the regulation of root system morphogenesis and the development of autophagy. The effect of the nitrogen oxide on the plant is characterized by diversity, which is due to the formation of physiologically active metabolites of NO, its interaction with various involved molecular targets, etc. [17, 24]. Nitric oxide can act both as a protector (antioxidant protection) and as a generator of free radicals. It is known that NO plays a key role as a signaling molecule under the action of various biotic and abiotic stress factors. The involvement of NO in the mechanisms of plant adaptation to microgravity, including the mediated participation in autophagy, remains unclear. The goal of this study was to investigate the role of NO in the regulation of seedling growth and the development of autophagy under simulated microgravity using exogenous NO donors and scavengers. The main results of these studies were published earlier [2, 23, 24].

In particular, it was found that SNP (as NO donor) at concentrations from 100 to 1000 μM had a stimulating effect on the growth of seedlings and increased their resistance to clinostating. Treatment of seeds with PTIO (NO scavenger) had a negative effect (root growth retardation) in dose-dependent manner. Under the influence of exogenous NO, a more intense formation of root hairs was observed in the differentiation zone of A.thaliana roots. Treatment with cPTIO led to the opposite effect — inhibition of the initiation and growth of root hair primordia, both in control and in clinostated seedlings. These results indicate the involvement of NO in the processes of root development. As for the mechanisms of action, NO can interact with other hormones, such as auxin, and the effect depends on their balance, including in the formation of gravitropic bending of the root [8, 27].

2.7. Determination of the intracellular content of nitrites (NO$_2^-$) and localization of NO in root apex tissues. The NO content was determined according to standard methods with some modifications [24, 30]. The method is based on the quantitative determination of nitrites using the Griess reagent after the formation of nitrite from endogenous NO. The concentration nitrites (μg/ml in crude substance) was determined in 6- and 9-day-old seedlings of A. thaliana, the seeds of which were treated with 500 μM SNP or cPTIO. The obtained results indicate an increase in the level of endogenous NO by 1.5 times in control and by 1.8 times — in clinostated plants, whose seeds were pretreated with SNP (Fig. 3). On the 9th day of cultivation, the content of NO gradually decreased, which may be the result of the consumption of the pool of stimulated NO or indicate the formation of

![Fig. 3. Endogenous NO content in A. thaliana seedlings after clinostating and SNP/cPTIO treatment](image-url)
Fig. 4. Localization of NO in the epidermal cells of the root apex and root hairs of *A. thaliana* on the 6th day of cultivation (a). Scale bar: 100 μm. Effects of SNP/cPTIO treatment on fluorescence intensity in root apex epidermal cells (b). Staining: DAF-FM DA [24]

plant adaptation to the influence of altered gravity. Treatment with cPTIO led to a decrease in the content of endogenous NO, both in control and clinostated plants. Moreover, NO production induced by clinostating was inhibited by NO scavenger treatment.

To determine the tissue localization of NO the fluorescent dye 4-amino-5-methylamino-2', 7'-difluorescein (DAF-FM DA) was used. As a result, more intense fluorescence was detected in epidermal cells and root hairs (Fig. 4) [24]. To confirm these data, we treated of *A. thaliana* seeds with SNP or cPTIO. Treatment of seeds with SNP led to an increase in the intensity of fluorescence in analysed cells, which indicates an increase in the level of NO, statistically significant in clinostated samples, and after treatment with cPTIO — to a decrease of this index in epidermal cells and root hairs (Fig. 4) [24].

The stimulating effect of NO may be related to the interaction between NO and ROS, which enhances redox homeostasis and increases the antioxidant capacity of cells [5]. Microgravity can increase both the activity of antioxidant enzymes and the content of glutathione, which is an important mechanism in the cell’s defense system against oxidative stress. Microgravity affecting the level of NO formation, increase NO content and NOS activity, as well as reduce its buffering capacity [29], which can negatively affect cells, for example, induce apoptosis [3].

2.8. The influence of nitric oxide on the development of autophagy in the cells of the root apex of *A. thaliana*. Visualization of autophagosomes in root cells was performed starting with 6-day-old seedlings, when induction of autophagy was observed (Fig. 1). As noted, under the conditions of clinostating, the intracellular number of autophagosomes in seedlings first increases, and then their gradual decrease occurs. To find out the role of NO in the development of autophagy, we also used a donor and scavenger of NO and found that treatment with a NO donor leads to an increase in the development of autophagy in the epidermal cells of the roots of clinostated plants. Treatment with cPTIO slightly inhibited the growth of seedlings, and this effect was enhanced when plants were clinostated, including an increase in the accumulation of autophagosomes in root epidermal cells.
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Possible molecular connection between NO-signaling and autophagy under stress conditions was found. In plants, as known, the intracellular level of S-nitrosoglutathione (GSNO), the main biologically active type of NO, is controlled by GSNO-reductase (GSNOR), which is a main regulator of NO signaling. NO-mediated S-nitrosylation can induce selective autophagy in A. thaliana in response to hypoxia [30]. S-nitrosylation of GSNOR1 at Cys-10 induces its local conformational changes that facilitate the interaction of GSNOR1 with ATG8. After binding to ATG8, GSNOR1 compartmentalized into autophagosomes and degraded [30]. Thus, this mechanism demonstrates the physiological dependence of selective autophagy on S-nitrosylation-induced GSNOR1 under stress conditions, thereby establishing a possible molecular link between NO signaling and autophagy.

3. CONCLUSIONS

The obtained results, which confirm the functional role of α- and β-tubulin genes in the development of stress-induced autophagy, create an initial platform for further investigation of the cellular functions of the different genes of ATG8 and their interaction with other cellular components involved in the development of autophagy under microgravity. Using effective donors and scavengers of NO made it possible to clarify the role of NO in mediating the action of such a stress as microgravity, and the obtained results will be used in the development of approaches to increase the plant adaptation to their cultivation during space flight. The obtained data can be used to solve the problems of growing plants in closed systems during long-term flights.

The work was carried out with the financial support of the project “Development of the concept of regulation of development and stress resistance of plants for their adaptation to the conditions of space flights by involving cell-biological resources” of the target complex program of the National Academy of Sciences of Ukraine on scientific space research for 2018—2022 (state registration number 01118U003742).

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З'ясування клітинних механізмів залучення автофагії у адаптацію рослин до умов мікрогравітації

Показано, що умови клиностатування індукують розвиток автофагії без збільшення індексу програмованої клітинної загибелі (ПКЗ) в епідермальних клітинах апексів коренів проростків A. thaliana. Після фази активізації автофагії настає її регуляторне послаблення, що правдоподібно свідчить про адаптивні зміни до умов клиностатування. Індукація автофагії корелює з підвищенням рівнів експресії генів atg8, частина з яких (atg8e та atg8i) може бути залучена до реалізації автофагії саме в умовах мікрогравітації. Проаналізовано транскрипційну активність генів цитоскелету, залучених до реалізації стрес-індукуваної автофагії, зокрема генів α- і β-тубуліну. Виявлено спільну експресію генів α- і β-тубулінів та atg8 в умовах симульованої мікрогравітації. Дані результати ілюструють роль цитоскелету при розвитку індукуваної мікрогравітацією автофагії та дають можливість виявити специфічні до цього виду стресу гени.

Індукцію автофагії та ПКЗ досліджували за дії гамма-опромінення як фактора космічних польотів, супутнього мікрогравітації, а також в умовах комбінованої дії гострого опромінення і клиностатування. Гамма-опромінення в дозах, еквівалентних таким в кабіні космічного корабля (1—6 Гр), індукувало дозозалежні зміни топології та цитогенетичного стану апікальної меристеми кореня, а також незначно пригнічувало ранній розвиток рослин. У меристемі збільшувалась гетерогеність, збільшувались показники ПКЗ, переважно проліферативної загибелі та автофагії. При комбінованій дії гамма-опромінення (2 Гр) і клиностатування шільність аутофагосом у клітинах коренів шестидобових проростків збільшувалась (24 год після опромінення), а через 4 доби зменшувалась порівняно з неопроміненим контролем.

Обробка насіння A. thaliana донором NO стимулювала розвиток рослин, підвищувала вміст ендогенного NO у тканинах кореня та стійкість рослин до клиностатування. В умовах клиностатування, у порівнянні з контролем, оптимум концентрації NO зменшувався, можливо, за рахунок внеску NO у генерацію АФК. Негативний ефект дії скавенжера NO на ріст проростків посилювався клиностатуванням, включаючи збільшення накопичення автофагосом в епідермальних клітинах. Ці дані вказують на те, що ендогенний вміст NO є важливим компонентом внутрішньоклітинних сигналних механізмів, залучених у відповідь рослинних клітин на імітовану мікрогравітацію, зокрема механізми індукації автофагії. Обробка насіння донором NO позитивно вплинула на розвиток проростків, зокрема збільшила вміст NO у тканинах кореня, зменшила індекс ПКЗ, а також позитивно вплинула на розвиток рослин в умовах клиностатування.

Ключові слова: автофагія, гени atg8, гени α- і β-тубулінів, оксид азоту, адаптація, клиностатування, гамма-опромінення, Arabidopsis thaliana