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X-RAY EXPOSURE TO THE STRESS RESPONSE FROM *Ri*-TRANSFORMED REGENERANTS OF *DIGITALIS PURPUREA L IN VITRO**

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Abstract. *Digitalis purpurea L. is the source of a number of biologically active substances used for medical purposes. Agrobacterium rhizogenes-transformed plants show changes in the secondary metabolism. The influence of acute exposure to ionizing radiation on the modification of the pigment system and accumulation of flavonoids in Ri-transformed regenerated plants in vitro was studied. A week after the exposure to the stressor, changes in the number of chlorophylls, carotenoids and flavonoids were observed. A month later, the amount of carotenoids and the ratio of chlorophyll a/b decreased, which indicated a decrease in the photosynthetic capacity of the irradiated plants. The synthesis of flavonoids was reduced as well. But in the second passage, we saw an increase in all the parameters studied in relation to the non-irradiated samples. It showed the development of a long-acting adaptive response of plants. A nonlinear dose dependence of acute X-ray irradiation influence on in vitro D. purpurea was shown. The transformed plants were found to be more resistant to the damaging effects of radiation. We reasoned that the exposure to ionizing radiation in a certain dose interval could be used to stimulate the accumulation of biologically active substances of in vitro D. purpurea.*

Key words: *Digitalis purpurea L., Ri-transformed regenerated plants, ionizing radiation, pigments, flavonoid*

1. INTRODUCTION

Digitalis purpurea L. is a valuable medicinal plant from the *Plantaginaceae* family and a source of glycosides that are used to treat cardiovascular diseases. An important task is to induct the plant into culture for developing the promising method of biotechnological production of medicines. The implementation of this task involves the development of plant cultivation and cell culture techniques that would allow achieving the increase of effective biomass with high specific concentration of pharmaceutically valuable glycosides. Previous studies showed that the plants obtained through regeneration from hairy roots, which had previously been transformed with a virulent strain of *Agrobacterium rhizogenes*, had the set of necessary properties [1; 2]. Such *Ri*-transformed regenerated plants were characterized with the increasing synthesis of the secondary metabolites due to the integration of *Ri*-plasmid T-DNA *rol*-genes of *Agrobacterium* into the plant genome with their next activation and gene expression [2]. However, in the case of *D. purpurea,* the synthesis of particular metabolites, cardioglycosides, has to be stimulated. The content of these glycosides in leaves correlates with both the flavonoids' concentration and photosynthetic activity [3]. The latter depends on the photosystems' state and the ratio of the total chlorophyll *a* and *b* content. At the same time, it is known that the expression of the *rolC* gene in the transformed plant cells reduces the concentration of chlorophyll primarily due to form *a*, which is necessary for efficient photosynthesis [2]. We have proposed to solve this problem by using the low-ionizing radiation exposure of *Digitalis* regenerants that would increase the chlorophyll content in the stimulation dose-range [4; 5]. In addition, the exposed plants are under the physiological stress due to the depletion of the antioxidant systems, which can lead to increasing protective flavonoid and glycoside synthesis due to biochemical pathways of flavonoid anabolism's activation. In order to test the proposed hypothesis, we conducted a series of experiments with radiation exposure on both intact plants and regenerants and then compared the physiological and biochemical parameters in the two variants of the experiment.

2. MATERIALS AND METHODS

The transformation of root explants, the *D. purpurea* regeneration from hairy roots culture and the subsequent cultivation *in vitro* was carried out as described in [6]. The strain *Agrobacterium rhizogenes ATCC15834* was used for the transformation. The 4-week-old intact and regenerants plants were irradiated under X-ray RUM-17 (National Cancer Institute, Kyiv, Ukraine) using doses 1, 2, 3, 5, 10 and 20 Gy with dose rate 1.27 Gy/sec and X-ray energy of 180 keV (15 plants, both variants for each radiation dose, including two non-exposed controls). Absorbed dose was about 95% of exposure dose. The stimulation dose-range was determined according to data about germination of radiation-exposed seeds and biomass accumulation of the *D.purpurea* aboveground part [7]. One week after

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the exposure, the water content and the mitotic index in the root apical meristem were measured using a standard technique [8]. Also, the content of chlorophyll *a* and *b* and both flavonoid and carotenoid amount in leaves were obtained in mg per 1 g of dry weight. Then, the explants were transplanted to a fresh MS medium and cultured for a month before the next measurement of indexes. Thus, the data for three consecutive passages for intact plants and regenerates were obtained. Pigments and flavonoids were extracted with 70% ethanol. The concentrations of the chlorophylls and carotenoids were detected with the spectrophluorimeter Fluorat-02-Panorama (Russia) under photometric mode with wavelengths 649 nm and 665 nm (for chlorophylls) and 470 nm (for carotenoids). The total flavonoid content was detected using the complex-formation reaction with $AlCl_3$ under 415 nm [9]. The complex of rutin with $AlCl_3$ was used as an etalon for digestion comparison. So far, we have analyzed one experimental data of the three series. Data processing was carried out using Microsoft Office Excel XP ("Microsoft", USA). The averaged indexes and STDs for each variant are shown in the figures. For the comparison of sample averages, the non-parametric U-Mann-Whitney criterion was used. The quantitative connection between radiation-induced different biochemical parameters variation was assessed with the index of Spearman's rank correlation coefficient.

3. Results and discussion

Acute radiation exposure increased the water content in both intact plants and *Ri*-transformed regenerated plants in a week after the exposure in the tested dose range (except for the 5 Gy-dose-exposure). Unexposed plants from both variants had the same water content indicator - 77-80%. In the second and third passage, the plant water content of all groups and variants was the same - 90-95%. Nevertheless, the water content of regenerants exposed with 5 Gy-dose decreased from 86% to 78%. It is important to note that under all other exposure doses, the positive dynamics of hydration was demonstrated in a month or two after the irradiation. Thus, the prolonged inhibitory effect was only shown in the transformed plants under 5 Gy-dose-exposure. The stimulating effect of acute X-ray radiation exposure on *in vitro* plants turned into short-term. Thus, the mitotic index in the root apical meristem was chosen as the other criterion for the physiological plant state. The plant roots exposed under 10 and 20 Gy were excluded from the analysis, because these radiation doses caused the mitosis delay. The stimulated cell division in the first passage of the intact plants was indicated with 1-5 Gy-radiation exposure, but the indexes came back to normal in two months after the irradiation. The increased mitotic index (compared with the control one) was preserved only for non-transformed *D.purpurea* in 5 weeks after the X-ray-exposure with 1 Gy-dose (the second passage). The exposure with up to 5 Gy-dose led to the opposite effect – decreasing the mitotic index according to control for regenerated plants and genetically transformed roots. The mitotic index is also different for non-exposed control variants – limiting its analytical value as a measure to compare the stability of intact plants and *Ri*-transformants to ionizing radiation exposure. The mitotic activity of transformants' meristems (both the roots' culture and regenerated roots) is higher, that is why they have less radioresistance. Moreover, the cell division rate in the con of origin may reflect various processes and does not necessarily characterize the plant state as a whole organism. The water content is a fairly reliable indicator of the current functional plant state. However, it is too variable for working with small samples and does not reflect prolonged metabolic changes. Therefore, to estimate radiation exposure, we decided to use pigment indexes: the total content of *a* and *b* chlorophylls (C*a*+C*b*) in leaves equivalent to 1 g of dry weight; the ratio C*a*/C*b*, and (C*a*+C*b*)/C*k*, where C*k* – carotenoids' content in leaves, mg/g. Radiosensitivity can be estimated by expressing the average group absolute index in % to non-exposed control. Thereat, the physiological plant state is most closely correlated with the index C*a*/C*b*, especially (C*a*+C*b*)/C*k* [9]. Dose curves for the parameters are shown in Fig. 1. The lower the parameter of C*a*/C*b* is, along with the (C*a*+ C*b*)/C*k* one, the lower the activity of the photosystem I is and the more carotenoids are synthesized over the necessary amount for the protection of the photosynthetic structure, the more expressed the physiological stress is. The obtained data indicated the maintenance of stress status for both intact plants and regenerants during two months after the radiation exposure under 10 and 20 Gy. The dose-curve analysis (Fig.1) indicates that *Ri*-transformed regenerated plants are much more resistant to radiation exposure than non-transformed plants. The observed effect was reduced up to the 5th week after the exposure (the second passage) and totally disappeared up to the 9th week (the third passage). The obtained results indicate that both the intact plants and *Ri*-transformed regenerated plants remained in physiological stress for two months after the 10 and 20 Gy dose-exposures. Such data mean that the possibility of radiation-induced repair of *in vitro D. purpurea* under the indicated doses is decreased. Also, the stimulation of the photosystem I in regenerated plants under 1 Gy dose-exposure is observed long-time after the exposure (superreparation). The modification of the normal (related to control) pigment system state due to the radiation exposure effect is accurately observed with dose curves (Fig. 2). The inhibition effect is shown under 10 and 20 Gy dose-exposures. In low-dose range (1–3 Gy), the active stimulation of chlorophylls and carotenoid synthesis is noticed. Moreover, the stimulating doses for *Ri*-transformants are higher on 2 Gy, that is, it may be connected to their higher radioresistance. The several-fold increase of *a* and *b* chlorophyll content in the leaves of the transformed plants under 3 Gy dose-exposure concerns not only the inhibition of constitutive chlorophyll degradation, but also its biosynthesis activation. It can be connected to the rapid increase of the carotenoid content and the decrease of the flavonoid part. The estimation shows a positive tendency between carotenoid content in leaves and index C*a*/C*b* (*rho*=0.71 for intact plants and *rho*=0.57 for regenerants; $p<0.05$), that confirms the protecting role of the carotenoids to photosystem I. It is interesting that non-exposed *Ri*-transformants in the first passage include much more chlorophylls than intact plants, due to the higher chlorophyll *b* content. Alternatively, their leaves have 2 times more carotenoids per mass index and to 10% less flavonoids than the leaves of non-transformed *D.purpurea* plants. However, at the end of cultivation, the differences between all three types of metabolites "change their sign" that is explained with the expression of *rol*-genes in the cells of the transformed regenerants [2].

Figure 2 indicates that the radiostimulation of flavonoid synthesis exists only under radiation exposure of *Ri*-transformed regenerants of *D. purpurea* with stress-causative doses 2 and 5 Gy (Fig. 1, B). It is also noticed that there is an inverse dependence (*rho* = -0.64; $p<0.05$) between the flavonoid content in leaves a week after the exposure and the stress index (C*a*+C*b*)/C*k*. It may be suggested that the flavonoid synthesis in *Ri*-transformed plants is activated with physiological stress due to radiation exposure. There is no significant correlation between these two indexes in intact *D. purpurea*.

According to this fact and the differences between the variants with different flavonoid and carotenoid content in non-exposed leaves, it may be suggested that the intact plants and *D. purpurea* transformants use various adaptation strategies to oxidative stress based on the activation of the antioxidant protective system. But the response of intact plants is associated with carotenoids and *Ri*- transformed regenerated plants – with flavonoids that may be due to *Agrobacterium*-mediated transformation.

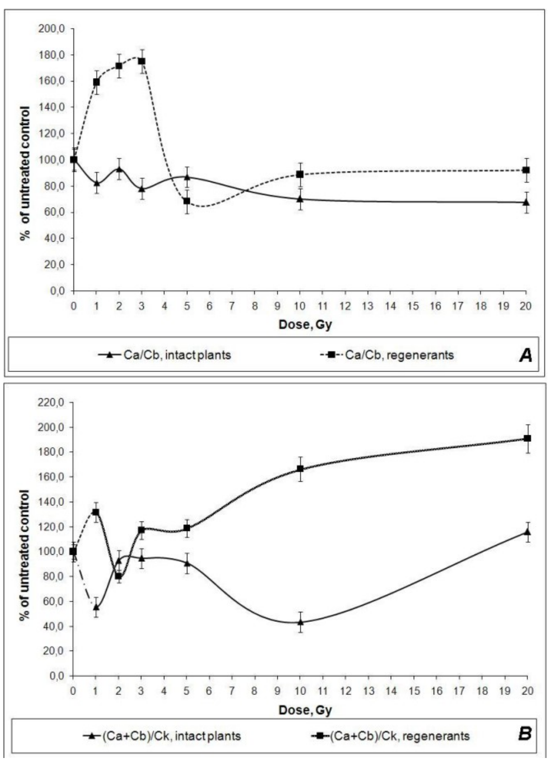

Figure 1. Dose dependence of the ratio of the content of chlorophyll *a* to the content of chlorophyll *b* **(A)** and chlorophylls (*a*+*b)* to the total carotenoids **(B)** in leaves of *D. purpurea* a week after irradiation

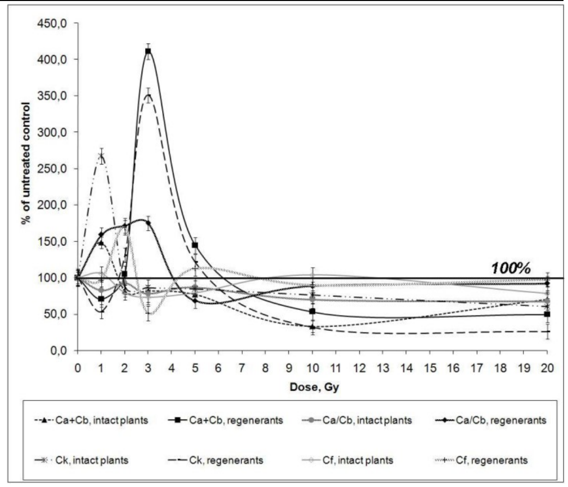

Figure 2. Dose dependence of total chlorophyll content C*a* + C*b*, the ratio C*a*/C*b*, total carotenoids C*k* and total flavonoids C*f* in the leaves of *D. purpurea* a week after irradiation

4. Conclusions

The effects of acute X-ray-radiation exposure to the pigment system and flavonoid synthesis of *in vitro Digitalis purpurea* intact plants and *Ri*- transformed regenerated plants were shown. The achieved radiobiological effect had a transitive character and depended on dose range 1–20 Gy. The pigment system of transformed regenerants showed a greater resistance to radiation exposure compared to intact plants. The possibility of using low-ionizing radiation exposure for synthesis stimulation of biologically active molecules – chlorophylls, carotenoids, flavonoids in the *in vitro* tissues of the *Ri*-transformed *D.purpurea* was shown.

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