ISSN 1561-8889. Космічна наука і технологія. 2010. Т. 16. № 2. С. 75–80.

UDC 579.65

O. P. Burlak[1], O. V. Lar[1], I. S. Rogutskyy[2], B. A. Danilchenko[2], O. M. Mikheev[3], I. Ye. Zaets[1], J.-P. de Vera[4], B. H. Foing[5], N. O. Kozyrovska[1]

[1] Institute of Molecular Biology&Genetics of NASU, Kyiv, Ukraine
[2] Institute of Physics of NASU, Kyiv, Ukraine
[3] Institute of Cell Biology& Genetic Engineering of NASU, Kyiv, Ukraine
[4] Institute of Planetary Science, DLR, Berlin, Germany
[5] ESA/ESTEC/SRE-S, postbus 299 NL-2200 AG, Noordwijk, The Netherlands

A BACTERIAL CONSORTIUM ATTENUATES THE LOW-DOSE GAMMA-IRRADIATION EFFECT IN KALANCHOE PLANTLETS

The ability of plants to protect themselves against ionizing radiation is limited. One of the ways how to alleviate irradiation consequences in plants is to use plant-associated bacteria for inoculation. Two defined plant growth promoting bacterial strains were used for inoculation Kalanchoe daigremontiana plantlets before acute irradiation with γ-quanta (^{60}Co). The lethal γ-rays doses were 3.0 kGy for Klebsiella oxytoca IMBG26, and 500 Gy for Paenibacillus sp. IMBG156. The bacteria expressed the increase of the pelX promoter activity after sublethal dose irradiation. The pelX promoter activity that was measured as activity of β-galactosidase of the pelX::lacZ fusion in K. oxytoca (pGalP) was 0,88 mkM/ml·min after exposure to 2.0 kGy, e.a. 80 % of the control (untreated) bacterial activity, although the irradiated bacterial population comprised 1.25 % of control one. Integrated index of plantlets development which was relied on both root number and root length reflected fluctuations in metabolic processes in irradiated plantlets without treatment with bacteria. Stabilizing stress-reactions occurred during 10 days after irradiation at different doses (30, 50, 70 Gy), however, index of growth (IG) remained at the level of 30—60 % to control plantlets. The effect of irradiation on kalanchoe plantlets was relieved by bacteria at doses of 30 and 50 Gy, moreover, IG was observed at levels of 500 and 200 %, respectively. A 30 Gy dose was obviously stimulating for K. daigremontiana plantlets. Intense root elongation, instead of development of new coronal roots, led to fast adaptation to stressful conditions and normalization of metabolic processes in kalanchoe plantlets. However, integrated index showed inhibition of both inoculated and non-inoculated plantlets development after getting a 70 Gy dose.

INTRODUCTION

Plants have adapted to respond to abiotic stressors at the molecular, cellular, physiological, and biochemical levels, enabling them to survive. It is suggested that Ionizing Radiation (IR) tolerance in plants relied not only on general but species-specific defense systems [5]. Exposure plants to a low dose rate of IR leads to an efficient induction of anti-oxidant enzyme activities involved in plant protection against reactive oxygen species [24]. A DNA hypermethylation that prevents genome instability is viewed as a defence strategy of plants, allowing survival in the extreme environment [9]. Plant cell protein, as well as storage proteins play roles in plant adaptation mechanism to radioactivity [5, 21]. Various plant species differ in sensitivity to radiation, depending on efficiency of defense mechanisms. For example, acute irradiation (60 Gy) of pine (*Pinus sylvestris*) resulted in death of pine trees near the Chernobyl atomic power station [1]. By contrast, the lethal dose for Arabidopsis was estimated to be more than 150 Gy [8].

To relieve the hazardous effect of IR in plants might involve the use of microorganisms [20]. The extremely radiation-resistant species of bacteria can survive acute exposures to ionizing radiation and can grow under chronic IR (60 Gy/hour) [14]. For example, *Deinococcus radiodurans* can survive levels of IR (10 kGy) that induce approximately 100 DNA double-strand breaks (DSBs) per genome, whereas *Shewanella oneidensis* is killed by levels of IR (0.07 kGy) that result in 1 DSB per genome. Microbes have evolved several mechanisms to tolerate IR. M. Daly with co-authors (2004) suggested that in resistant bacteria the degree of resistance is determined

not by efficient DNA repair system, but by the level of oxidative protein damage caused during irradiation. Their hypothesis of extreme IR resistance in *D. radiodurans* is that non-enzymic Mn(II) complexes present in resistant bacteria protect proteins, but not DNA, from oxidation during irradiation, with the result that conventional enzyme systems involved in recovery survive and function with far greater efficiency than in sensitive bacteria [3, 4]. The proposed mechanism of extreme IR resistance requires a high intracellular Mn/Fe concentration ratio. Except genes involved in manganese import, the genes coding for nutrient import and DNA repair are likely important for survival and adaptation of deinococci to its hostile environment [6]. Other radiation-resistance mechanisms are in relationship to protective systems such as antioxidative enzymes which involve peroxidase, catalase, and superoxide dismutase [13]. These bacterial enzymes represent an important defensive system against reactive oxygen species induced by hydrogen peroxide and/or radiation. Several bacteria possess a substantial amount of non-enzymatic systems (e. g., vitamins A and E) which contribute to the resistance phenomenon against the deleterious effects of radiation damages.

Two dual strains of bacteria, *Klebsiella oxytoca* IMBG26 and *Paenibacillus* sp. IMBG156, are known as plant growth promoting bacteria [10] which relieve the effect of heavy metals on the plant [23]. The objective of this study was to define whether the consortium of plant-associated bacteria is capable to alleviate γ-irradiation effect on kalanchoe plantlets.

MATERIALS AND METHODS

Bacteria and media. Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown in following nutrient media**:** *Paenibacillus* sp. in M9 [16] at 28 °C 24 hours, *E. coli* and *K. oxytoca* in LB [16] at 37 °C during 18 hours. Rifampicin (100 mg/ml) and ampicillin (50 mg/ml) were added to nutrient media when appropriate. For acute IR (^{60}Co) exposures to doses of 30, 50, 70 Gy, late logarithmic-phase cultures were used. Colony-forming units (CFU) were determined by plate assay, using culture dilutions and spreading on appropriate agar plates. Three independent irradiation treatments of the same kind were performed and served as biological replicates for determining irradiation resistance profiles.

DNA manipulations (the plasmid DNA isolation, DNA restriction with endonucleases and ligation, DNA fragment isolation from agarose gel) were performed according to the recommendations given by Sambrook et al. [19]. *Sma*I and *Pst*I purchased in Fermentas (Lithuania). Plasmid transformation was performed as recommended by Nishimura et al. [18]. A search for promoter sequences was carried out with the use of the program Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html).

Biochemical analyses. Beta-galactosidase activity was measured as recommended by J. Miller [16]. Inductors Glycerine, Glucose, Na-Polygalacturonate were added when appropriate at a concentration of 0.2 %. The plant extract was prepared as described in [12].

Table 1. **Strains and Plasmids Used in the Study**

Strain or Plasmid	Genotype or Phenotype	Origin
Escherichia coli JM109	*rec*A1, *end*A1, *gyrA96*, *thi*, *hsdR17*, *supE44*, *relA1* λ^-, Δ(*lac-pro*AB), F', *traD36*, *proAB*, *lac*I^{Q}ZΔM15	Institute collection
Klebsiella oxytoca IMBG26 (VN13)	Rif^r, Ap^r, wild type	Institute collection
Klebsiella oxytoca IMBG27	PelX$^-$, Ap^s	Institute collection
Paenibacillus sp. IMBG156	wild type	Institute collection
pLC28P	the 3-kb *Eco*RV- *Sac*I fragment, carring the p*elX* gene; Ap^R	Institute collection
pCB192	promoterless *lac*Z	Institute of Microbiology, Bayreuth University, FRG
pGalP	*pelX*::*lac*Z	Institute collection

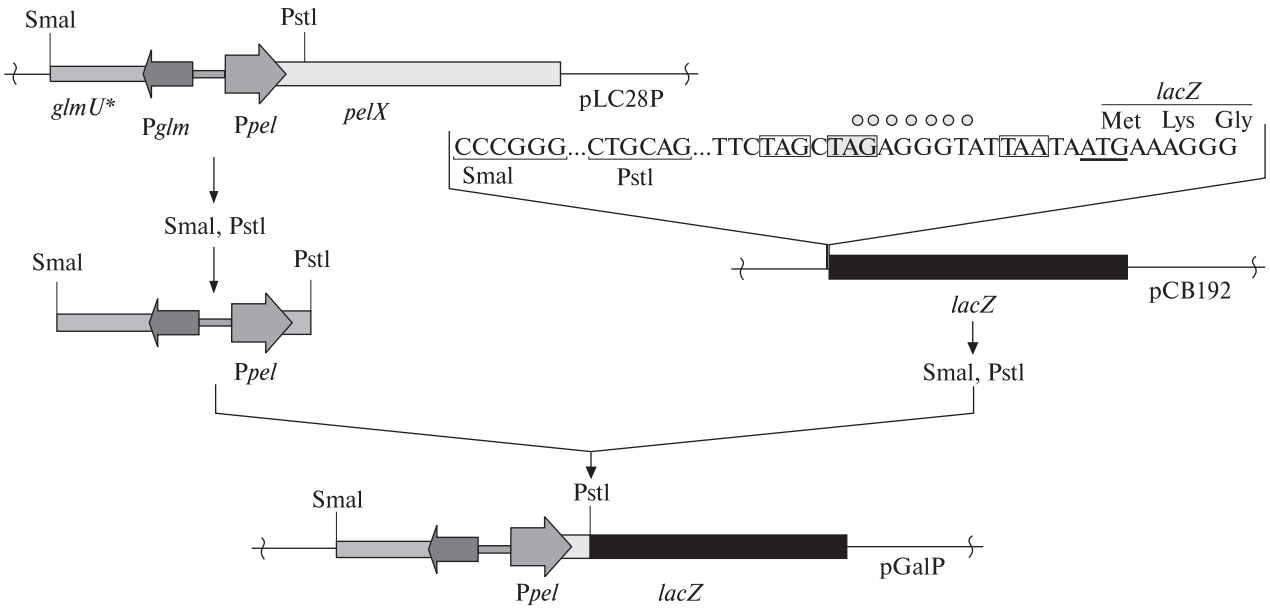

Fig. 1. The scheme of fusion of the *pel*X gene promoter with the *lac*Z gene coding part. Stop codons that terminate translation are marked with frames, the ribosome binding site are marked with circles. The beginning of the *lac*Z gene and the translated amino acid sequence is displayed. The fusion is used to measure the β-galactosidase activity in irradiated bacterial population

Plantlets inoculation was performed with the 1:100 diluted overnight cultures mixed in equal aliquots. One-size two-leaves plantlets where dropped into the mixed culture (10^6 CFU/ml) for 20 min a day before irradiation.

Plantlets irradiation was performed on MPX-γ-25M (Institute of Physics of NASU) with the use of doses of 30, 50, and 70 Gy. Plant inoculation was done with *K. oxytoca* IMBG26, and *Paenibacillus* sp. IMBG156 a day before irradiation. After irradiation the plantlets were put onto 2.0 % agar medium and photographed every day under standard conditions within 20 days. The root length was measured in pixels using the tpsDig v.2 program. The increase of both root length and number of roots under some dose *n* was determined by Index of Growth (IG): $IG_n = N_i/N_0$, where N_i is number of roots in a day *i*, N_0 denotes number of roots in the first day. IG of control untreated plants (IG*c*) was taken as 100 %, and IG of experimental plants (IG_n) corresponded with control: $(IG_n / IG_c) \cdot 100\ \% = K_n$.

The statistical analysis of the significance of differences between means was based on Student's *t*-test ($P < 0.05$).

RESULTS AND DISCUSSION

A translation fusion pelX::lacZ construction. We constructed the translation fusion of the *pel*X promoter with the coding part of the *lac*Z reporter gene to monitor the exopectate lyase gene expression by biochemical analysis (Fig. 1). The *pel*X was identified, cloned, and sequenced earlier [11]. A promoter part was determined based on a full gene nucleotide sequence inserted in *pLC28P*, and the *Sma*I-*Pst*I fragment was restricted and transferred into *pCB192*, carring the promotorless *lac*Z gene. The resulted *pGalP* contained both three stop-codons that terminated translation within three reding frames and a ribosome-binding site, allowing the *lac*Z gene translation (see Fig. 1). So far mRNA was being transcribed from the *pel*X gene promotor and translated into β-galactosidase. Resulted *pGalP* was introduced in both *E. coli* JM109 and *K. oxytoca* IMBG27.

Comparative studies on expression of the *pel*X::*lac*Z fusion in *E. coli* and *K. oxytoca* using such inductors as a plant extract, sodium polygalacturonate, glycerine or glucose showed a low constitutive level of the *pel*X expression in *K. oxytoca,* as well incomplete derepression effected by these inductors.

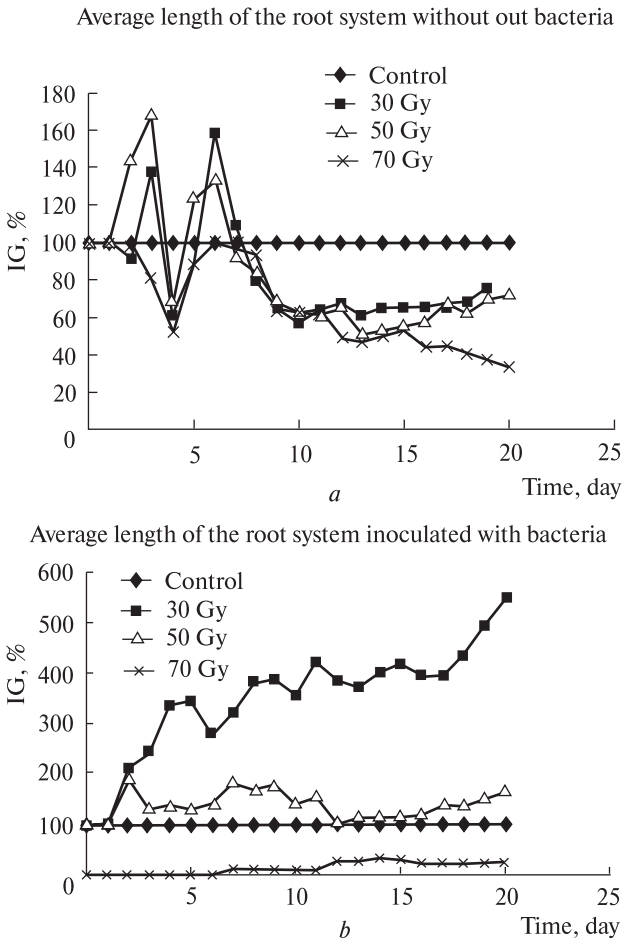

Fig. 2. The effect of acute doses of ionizing radiation (^{60}Co) on a state of primary root system of *Kalanchoe daigramontiana* without (*a*) and using plantlet inoculation with plant growth promoting bacteria (*b*) reflected with integrated tolerance index which comprises both the number and length of primary roots. IG (Index of Growth) reflects an increase of root numbers at the certain dose *n*: $IG_n = N_i/N_0$, where N_i is the root number at the estimated day, N_0 denotes the root number at the first day. For representation of all doses effect, IG_c of control plantlets is accepted as 100 %, other indexes corresponded as $(IG_n/IG_c)\cdot 100\ \% = K_n$

β-Galactosidase activities of the *pel*X::*lac*Z fusion in *E. coli* JM109 and *K. oxytoca* IMBG27 are represented in Table 2.

Irradiation of model bacteria. Acute irradiation with γ-quanta showed that the sublethal dose for *K. oxytoca* IMBG26 was 2 kGy, and for *Paenibacillus* sp. IMBG156 it was 300 Gy. As compared to other bacteria, selected species were not highly resistant to IR. After exposure to a sublethal dose the *K. oxytoca* (*pGalP*) *pel*X gene activity was 0.88 mkM/ml·min, e.a. 80 % of control (untreated) bacterial activity, although the irradiated bacterial population comprised only 1.25 % from control one (Table 3). These data may show an increased level of the *pel*X gene expression in irradiated bacteria as compared to untreated ones. The conclusion was done that a low-dose IR was not harmful for model bacteria.

Effect of bacterial inoculation on morpho-physiological parameters of irradiated plants. A state of root meristem as the most sensitive to external factors was taken into account for the evaluation of kalanchoe plantlets radiotolerance in a period of embryogenesis. After a low-dose gamma irradiation both a number and length of roots were measured, as well as average length of a root system taken as a ratio of average root length to average root number. As seen from Fig. 2*a*, a reaction of plantlets on irradiation without bacterial inoculation was not unimodal. In the next day after acute irradiation the plantlet roots showed an increased growth as compared to untreated plants, however, a sharp intensity of metabolic processes resulted in growth inhibition. At the end of the first week a small activation of metabolic processes was

Table 2. **β-Galactosidase Activity of the *pelX*::*lacZ* Fusion in *Escherichia Coli* JM109 and *Klebsiella Oxytoca* IMBG27**

Inductors, 0.2%	β-galactosidase activity, mkM/(ml·min)	
	E. coli (*pGalP*)	*K. oxytoca* (*pGalP*)*
Glycerine	27.55	3.21
Glucose	16.69	2.07
Na-Polygalacturonate	18.7	6.95
Plant extract	28.5	3.31

* The result was obtained when bacteria grew on antibiotic-containing medium as the indication for bacteria conferring plasmid.

Table 3. **β-Galactosidase Activity of Irradiated *Klebsiella oxytoca* (*pGalP*)**

Strain	*The* pel*X promoter activity*, mkM/(ml·min)	Average CFU/ml
K. oxytoca (*pGalP*) (control)	1.08	$4\cdot10^9$
K. oxytoca (*pGalP*) (irradiated; 2 kGy)	0.88	$5\cdot10^7$

seen again, however, it was below the control level, except a variant exposed to a 30 Gy dose. Integrated index of growth (IG), displaying dependence of average root length on doses of acute ionizing radiation, includes both parameters mentioned above, and seems to be more systemic in evaluation of IR effect on the plantlet. In this experiment, the integrated index reflected a fluctuation of metabolic processes in noninoculated plantlets which resulted in a stabilizing stress-reactions within 10 days after the irradiation; IG remained at the level from 30 to 60 % as compared with the control plantlets (see Fig. 2*a*).

In experiments on inoculations, *Paenibacillus* sp. IMBG156 and *K. oxytoca* IMBG26 were used for kalanchoe plantlets treatment. The effect of irradiation on kalanchoe plantlets was relieved by bacteria, however, this varied in dependence on an implemented dose (Fig. 2*b*). Integrated index showed inhibition of inoculated plantlets development after getting a 70 Gy dose. This IR dose appeared injured root cap meristem and inhibited root elongation. However, lower doses, both a 30 and 50 Gy doses, accepted by plantlets more easier after bacterial inoculation than by the control plantlets. Moreover, IG was observed at levels of 500 and 200 %, respectively. Intense root elongation instead of roots bearding led to fast plant adaptation to stressful conditions and normalization of metabolic processes in the irradiated plantlets. M. Melki and B. Sallami [15] tried to explain the stimulation of the root system after low-dose IR with the availability of more soil volumes to the plants to explore and therefore to support better water shortages by keeping more hydrated tissues and less damaged cell membranes at least during their first development stages. By all means, the volume of a root system plays a major role in plant survival under adverse conditions, and plant growth promoting bacteria activated this instrument of plant protection.

In this study the bacterial consortium alleviated the negative effect of gamma-irradiation on the plantlet root system development at doses of 30 and 50 Gy. Survival in the plant world depends on the ability to resist not only diseases, but also changes in the physical surroundings. There are several common elements responded on abiotic stressors in terrestrial plants, and repression of growth-related genes is one of them [7]. Model plant growth promoting bacteria increased the root length in irradiated kalanchoe and probably prevented repression of the genes related to plant growth. We may also speculate that acquisition of tolerance to 30 and 50 Gy doses in kalanchoe plantlets may be explained with induction of systemic tolerance to abiotic stressors by bacteria, however, this should be investigated in further studies. It is known that the rhizosphere microbes can protect plants against such environmental stressors, like heavy metal toxicity, salt, and drought via induction of systemic tolerance [22]. Reactive oxygen species (ROSs) are produced intracellularly in response to various stressors due to malfunctioning of cellular components, and have been implicated in many different signaling cascades in plants [17]. Protection against ROS is at the basis of every stress response, and bacteria may alleviate oxidative stress. Earlier we showed that stimulation of plant antioxidant system and phenolics production under assistance of plant-associated bacteria resulted in heavy metals tolerance in soybeans [23].

REFERENCES

1. *Arkhipov N. P., Kuchma N. D., Askbrant S., et al.* Acute and long-term effects of irradiation on pine (*Pinus sylvestris*) stands post-Chernobyl // Sci. Tot. Environ. — 1994. — **157**. — P. 383—386.
2. *Daly M. J.* Engineering radiation-resistant bacteria for environmental biotechnology // Curr. Opin. Biotechnol. — 2000. — **11**. — P 280—285.
3. *Daly M. J., Gaidamakova E. K., Matrosova V. Y., et al.* Accumulation of Mn(II) in Deinococcus radiodurans facilitates gamma-radiation resistance // Science. — 2004. — **306**. — P. 1025—1028.
4. *Daly M. J., Gaidamakova E. K., Matrosova V. Y., et al.* Protein oxidation implicated as the primary determinant of bacterial radioresistance // PLoS Biol. — 2007. — **5**. — P. 769—779.
5. *Danchenko M., Skultety L., Rashydov N. M., et al.* Proteomic analysis of mature soybean seeds from the Chernobyl area suggests plant adaptation to the contaminated environment // J. Proteome Res. — 2009. — **8**, N 6. — P. 2915—2922.
6. *de Groot A., Dulermo R., Ortet P., et al.* Alliance of proteomics and genomics to unravel the specificities of Sahara bacterium *Deinococcus deserti* // PLoS Genet. — 2009. — **5**, N 3. — e1000434.
7. *Dittami S., Scornet D., Petit J.-L., et al.* Global expression analysis of the brown alga *Ectocarpus siliculosus* (Phaeophyceae) reveals large-scale reprogramming of the transcriptome in response to biotic stress // Genome Biology. — 2009. — **10**. — R66.

8. *Kovalchuk I., Abramov V., Pogribny I., et al.* Molecular aspects of plant adaptation to life in the Chernobyl zone // Plant Physiol. — 2004. — **135**. — P. 357—363.
9. *Kovalchuk O., Burke P., Arkhipov A., et al.* Genome hypermethylation in *Pinus silvestris* of Chernobyl — a mechanism for radiation adaptation // Mutat. Res. — 2003. — **529**. — P. 13—20.
10. *Kozyrovska N., Negrutska V., Kovalchuk M., et al. Paenibacillus* sp., a promising candidate for development of a novel technology of plant inoculant production // Biopolymers and Cell. — 2005. — **21**, N 4. — P. 312—319.
11. *Lar O. V., Kovtunovych G. L., Kozyrovska N. O.* Cloning and analysis of the gene encoding pectate lyase, the *Klebsiella oxytoca* VN13 *pel*X // Biopolymers and Cell. — 2002. — **18,** N 5. — P. 417—422.
12. *Lar O. V., Kovtunovych G. L., Kozyrovska N. O.* A study of *Klebsiella oxytoca* exopectate lyase the *pel*X gene// Biopolymers and Cell. — 2005. — **21**, N 3. — P. 264—270.
13. *Le-Tien C., Lafortune R., Shareck F., et al.* DNA analysis of a radiotolerant bacterium *Pantoea agglomerans* by FT-IR spectroscopy // Talanta. — 2007. — **71**, N 5. — P. 1969—1975.
14. *Makarova K. S., Omelchenko M. V., Gaidamakova E. K., et al. Deinococcus geothermalis*: The pool of extreme radiation resistance genes shrinks // PLoS ONE. — 2007. — **2**, N 9. — P. 955.
15. *Melki M., Sallami D.* Studies the effects of low dose of gamma rays on the behaviour of chickpea under various conditions in Pakistan // J. Biol. Sci. — 2008. — **11**, N 19. — P. 2326—2330.
16. *Miller J. H.* Experiments in Molecular Genetics. — New York, Cold Spring Harbor Laboratory Press, 1972. — 432 p.
17. *Mittler R., Vanderauwera S., Gollery M., et al.* Reactive oxygen gene network of plants // Trends Plant Sci. — 2004. — **9.** — P**.** 490—498.
18. *Nishimura A., Morita M., Sugino Y. A.* Rapid and highly efficient method for preparation of competent *Esherichia coli* cells // Nucl. Acid Res. —1990. —**18**. — P. 6169.
19. *Sambrook J., Fritsch E. F., Maniatis T.* Molecular Cloning: a laboratory manual. — Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y., 1989.
20. *Sorochinskiy B., Kozyrovska N.* Biotechnological aspects of problem associated with phytoremediation of the environment from radionuclide pollution // Agrobiotechnologia. — 1998. — **2**. — P. 123—130.
21. *Sorochinskiy B., Prokhnevskiy O., Ruchko M.* Some mechanisms of somatic effects of irradiation indicated in plants from the 10 km zone of Chernobyl APS // Cytologyia i Genetika. — 1996. — **30**, N 4. — P. 15—19.
22. *Yang J., Kloepper J. W., Rye C. M.* Rhizosphere bacteria help plants tolerate abiotic stress // Trends Plant Sci. — 2009. — **14**. — P. 1—4.
23. *Zaetz I. E., Kozyrovska N. O.* Effect of a bacterial consortium on oxidative stress in soybean plants in cadmium-contaminated soil // Biopolymers and Cell. — 2008. — **24**. — P. 246—253.
24. *Zaka R., Vandecasteele C. M., Misset M. T.* Effects of low chronic doses of ionizing radiation on antioxidant enzymes and G_6PDH activities in *Stipa capillata* (Poaceae) // J. Exp. Bot. — 2002. — **53**, N 376. — P. 1979—1987.

Received October 1, 2009

О. П. Бурлак, О. В. Лар, І. С. Рогуцький,
О. М. Міхєєв, І. Є. Заєць, Ж.-П. де Вера,
Б. О. Данільченко, Б. Н. Фоїнг, Н. О. Козировська

БАКТЕРІЙНИЙ КОНСОРЦІУМ ПОСЛАБЛЮЄ ДІЮ ГАММА-РАДІАЦІЇ НИЗЬКОЇ ДОЗИ В ЛИСТКОВИХ БРУНЬКАХ КАЛАНХОЕ

Здатність рослин захищати себе від іонізуючого опромінення є обмеженою. Одним із засобів полегшити наслідки опромінення рослин є використання для їхньої інокуляції бактерій, асоційованих з рослинами. Для інокуляції листкових бруньок *Kalanchoe daigremontiana* були використані два види бактерій, якими обробляли рослини перед опроміненням γ-квантами ^{60}Co. Летальними дозами γ-опромінення для *Klebsiella oxytoca* IMBG26 були 3 кГр, а для *Paenibacillus* sp. IMBG156 – 500 Гр. Бактерії показали підвищення фізіологічної активності після сублетальної дози радіації. Активність *pel*X промотора, що вимірювалась як активність β-галактозидази гена *pel*X::*lac*Z, перенесеного в *K. oxytoca* (pGalP), була 0.88 мкм/(мл·хв) після опромінення дозою 2 кГр, що складає 80 % від контролю (неопромінена культура), хоча опромінена бактеріальна популяція становила лише 1.25 % від контрольної. Комплексний показник розвитку рослин, який визначали за числом і довжиною коренів, відображав коливання обмінних процесів в опроміненій рослині без обробки бактеріями. Стабілізація стрес-реакції відбувалася через 10 діб після опромінення при різних дозах (30, 50, 70 Гр), однак збільшення індексу росту (ІР) залишалось на рівні 30--60 % до контролю. Вплив опромінення на каланхое послаблювався бактеріями при дозах 30 і 50 Гр. Крім того, ІР спостерігався на рівні 500 і 200 % відповідно, а доза 30 Гр, очевидно, була стимулюючою для розвитку *K. daigremontiana*. Інтенсивне подовження кореня, замість розвитку нових мочкуватих корінців, призвело до швидкої адаптації до стресових умов і нормалізації обмінних процесів у каланхое. Разом з тим комплексний показник показав гальмування розвитку листкових бруньок каланхое після отримання дози 70 Гр.