

УДК 581.527.4+57.085.2(234.86)

## Rare and endemic plant species of Mountain Crimea flora: a morphogenic response to conservation in the gene bank *in vitro*

N. N. Ivanova<sup>1,3\*</sup>, V. A. Tsiupka<sup>1,4</sup>, O. V. Mitrofanova<sup>1,5</sup>, I. V. Mitrofanova<sup>2,6</sup>

<sup>1</sup>FSFIS “The Labor Red Banner Order Nikita Botanical Gardens – National Scientific Center of the RAS”,  
Nikitsky spusk St., 52, Yalta, 298648, Russia

<sup>2</sup>FSFIS Main Botanical Garden named after N. V. Tsitsin RAS, Botanicheskaya St., 4/1, Moscow, 127276, Russia

<sup>3</sup>E-mail: nnivanova2017@yandex.ru; ORCID iD: <https://orcid.org/0000-0001-7628-9646>

<sup>4</sup>ORCID iD: <https://orcid.org/0000-0003-3853-0210>

<sup>5</sup>ORCID iD: <https://orcid.org/0000-0002-4878-2828>

<sup>6</sup>ORCID iD: <https://orcid.org/0000-0002-4650-6942>

\* Corresponding author

**Keywords:** deposition, explant, *in vitro*, osmotic, rare endemic, retardant.

**Summary.** Biotechnological approaches have been actively used in recent years to preserve rare and endemic plant species under slow growth conditions. This is reached by lowering the temperature and light intensity, increasing the osmotic concentration, introducing retardants into the culture medium. For conservation, we used microshoot segments of *Lamium glaberrimum* (K. Koch) Taliev (Lamiaceae), *Crepis purpurea* L. (Asteraceae), *Scrophularia exilis* Popl. (Scrophulariaceae), *Silene jailensis* N. I. Rubtsov (Caryophyllaceae) cultured under standard *in vitro* conditions for 8–10 months. The explants were placed on ¼ MS culture medium supplemented with plant growth inhibitors: 0.2 g/L 2-chloroethyl-trimethyl-ammonium chloride (CCC) and 60 g/L sucrose. Conservation conditions were: the temperature 4–6 °C, light intensity 1.25–3.75 μM m<sup>-2</sup> s<sup>-1</sup>, photoperiod 16 hours. Screening of the morphometric parameters indicated high viability and a decrease in growth kinetics after 12 and 24 months of deposition in the gene bank *in vitro*. Along with the microshoot slow growth, the formation of adventitious shoots, leaves, and roots was observed.

## Редкие и эндемичные виды растений флоры Горного Крыма: морфогенетический ответ на сохранение в генбанке *in vitro*

Н. Н. Иванова<sup>1</sup>, В. А. Цюпка<sup>1</sup>, О. В. Митрофанова<sup>1</sup>, И. В. Митрофанова<sup>2</sup>

<sup>1</sup>ФГБУН «Ордена Трудового Красного Знамени Никитский ботанический сад – Национальный научный центр РАН»,  
ул. Никитский спуск, д. 52, г. Ялта, 298648, Россия

<sup>2</sup>ФГБУН Главный ботанический сад им. Н. В. Цицина РАН, Ботаническая ул., д. 4, стр. 1, г. Москва, 127276, Россия

**Ключевые слова:** депонирование, осмотик, редкий эндемик, ретардант, эксплант, *in vitro*.

**Аннотация.** Биотехнологические подходы активно применяются в последние годы для сохранения в условиях замедленного роста редких и эндемичных видов растений. Это достигается путем снижения температуры, интенсивности освещения, повышением концентрации осмотиков, введения в состав питательной среды ретардантов. Для депонирования использовали сегменты микропобегов *Lamium glaberrimum* (K. Koch) Taliev (Lamiaceae), *Crepis purpurea* L. (Asteraceae), *Scrophularia exilis* Popl. (Scrophulariaceae), *Silene jailensis* N. I. Rubtsov (Caryophyllaceae), культивируемых в стандартных условиях *in vitro* в течение 8–10 месяцев. Экспланты помещали на питательную среду  $\frac{1}{4}$  MS, дополненную ингибиторами роста: 0,2 г/л хлорхолинхлорида ССС и 60 г/л сахарозы. При депонировании температура составила 4–6 °С, интенсивность освещения 1,25–3,75 мкМ м<sup>-2</sup> с<sup>-1</sup>, фотопериод 16 часов. Проведенный скрининг морфометрических показателей показал высокую жизнеспособность и снижение кинетики роста в течение 12 и 24 месяцев депонирования в генбанке *in vitro*. Наряду с замедленным ростом микропобега наблюдали образование дополнительных побегов, листьев и корней.

## Introduction

Nowadays, an important part of the world biodiversity conservation is the possibility of plant resources depositing *in vitro*, which makes it possible to preserve long-time genetic collections of wild and cultivated plant species. Biotechnological approaches have been actively used in recent years to preserve rare, endemic, and endangered plant species listed in the republican Red Data Books and the Red Data Book of the Russian Federation under conditions of slow growth. This is reached by lowering the temperature and light intensity, increasing the osmotic concentration, and introducing retardants into the culture medium (Belletal, 2002; Engelmann, 2011). Rare endemic species require particular attention, since in nature their number constantly decrease due to the low degree of reproduction in nature growth conditions (Nikiforov et al., 2016; Whitlock et al., 2016; Nikiforov, 2018). Many endemic species are listed in the “Red Data Books of the Russian Federation (plants and fungi)” (2008) and “Red Book of the Republic of the Crimea (plants, algae and mushrooms)” (2015). Biotechnological methods make it possible to preserve valuable rare species, as well as single specimens *in vitro*, which are an integral part of the Global Plant Biodiversity Preservation concept (Sarasan et al., 2006; Cruz-Cruz et al., 2013; Engelmann, 2013; Novikova, 2013; Molkanova et al., 2018). One of the most promising ways for plant biodiversity conservation is the formation of gene banks *in vitro*. Nowadays, the Laboratory of Plant Biotechnology and Virology of the Nikita Botanical Gardens carries out complex studies of rare and endemic species of Mountain Crimea flora in the field of somatic embryogenesis and organogenesis induction, clonal micropropagation and subsequent long-term conservation *in vitro* at low positive temperatures (Mitrofanova et al.,

2020). In the process of plant deposition protocols development considering their genotype, along with exposure to low positive temperatures, a complex of osmotics and retardants were used, which slow down the growth of the studied plants under *in vitro* conditions (Mitrofanova, 2018).

Thus, plants, maintained under different culture conditions, retain both characteristics of their genotype and acquire some new features correspond with the conditions of cultivation and conservation. Thus, specimens preserved for a long time under *in vitro* conditions can have various morphological changes in microshoots and leaves, structural features of organs, which enable to determine the limits of the anatomical and physiological changes, reveal the dependence of the growth processes on the studied regenerants structure (Mitrofanova, 2018).

The objective of this study was to investigate the features of long-term conservation and identify morphological and anatomical features of some rare and endemic plant species after 24 months of deposition in the genebank *in vitro*.

## Materials

### Plant material

1. Microshoots of *Lamium glaberrimum* (K. Koch) Taliev (Lamiaceae), *Crepis purpurea* L. (Asteraceae), *Scrophularia exilis* Popl. (Scrophulariaceae), *Silene jailensis* N. I. Rubtsov (Caryophyllaceae) cultured under the standard conditions *in vitro* for 8–10 months.

### Reagents

1. Macro- and microelements, vitamins were used according to  $\frac{1}{4}$  MS (Murashige, Skoog, 1962).  
2. Agar-agar (Panreac, Spain).  
3. 2-chloroethyl-trimethyl-ammonium chlorid (CCC, 'Cycocel', AcrosOrganics, China).

4. Sucrose (Panreac, Spain).
5. Methylene Blue (3,7 Bis(dimethylamino) phenazathionium Chloride, Spectrum, China).
6. Phloroglucinol (1, 3, 5-Trihydroxybenzene, Merck, Germany).

#### Equipment

1. Laminar flow cabinet SC2 (“ESCO”, Singapore).
2. Steam sterilizer LAC 5060S (DAIHAN LABTECH, South Korea).
3. Culture vessels (150–200 ml volume).
4. QuartzBeads sterilizer (TauSteril, Italy).
5. Refrigerating chambers FKvsl 4113 (LIEBHERR, Austria).
6. Light microscope CX41 (Olympus, Japan) with SC 50 camera (Olympus, Germany) and Cell Sens Imaging Software version 1.17.
7. BIOTRON’s phytocapsules with a cool white light of fluorescent lamps (PhilipsTL, Japan).

### Procedures

#### Culture Media and Conditions

¼ MS medium (MS) supplemented with 9 g/L agar-agar, 30 g/L sucrose, 0.2 g/L 2-chloroethyl-trimethyl-ammonium chloride (CCC).

Control – ¼ MS medium supplemented with 60 g/L sucrose.

The pH of the medium was adjusted to 5.7 before autoclaving (120 °C 10 min).

Cultures were maintained under cool white fluorescent lamps (Philips TL, Japan) at light intensity of 1.25–3.75 µM m<sup>-2</sup> s<sup>-1</sup>, 16-hour photoperiod and temperature 4–6 °C.

#### Explants preparation

1. Segments without leaves 0.5–1.5 cm long were excised: *C. purpurea* (0.5 cm), *L. glaberrimum*, *S. exilis*, and *S. jaiensis* – 1.0 cm.

2. Explants were placed on ¼ MS medium for 12 and 24 months.

3. After 12 and 24 months conservation, the morphometric indexes of the objects were screened.

#### Morphometric parameters of explants maintained in vitro for 12 months

1. Morphometric parameters screening of the explants after 12 months deposition revealed that their viability on ¼ MS culture medium supplemented with 0.2 g/L CCC and 60 g/L sucrose at 4 °C was 98 % in *C. purpurea* plants, 95 % in *S. exilis*, *S. jaiensis* and *L. glaberrimum* (Table).

Table

Morphometric characteristics of the explants after 12 and 24 months conservation at temperature 4 °C on ¼ MS medium supplemented with 60.0 g/L sucrose and 0.2 g/L CCC

Terms of conservation, months	Shoot length, cm	Shoot thickness, µm	Number of leaves	Leaf thickness, µm	Number of adventitious shoots	Number of roots per shoot.	Root length, cm	Viability, %
<i>Crepis purpurea</i>								
12	0.88 ± 0.037	1033 ± 163	3.2 ± 0.20	159 ± 23	0	1.0 ± 0.0	1.6 ± 0.1	98
24	0.98 ± 0.01	1613 ± 209	3.75 ± 0.16	198 ± 26	0.88 ± 0.13	1.75 ± 0.25	2.5 ± 0.3	85
<i>Lamium glaberrimum</i>								
12	2.0 ± 0.0	1208 ± 215	2.2 ± 0.4	164 ± 82	0	0	0	95
24	2.28 ± 0.06	1215 ± 156	2.38 ± 0.18	209 ± 24	0	0	0	75
<i>Scrophularia exilis</i>								
12	1.42 ± 0.04	1230 ± 140	4.6 ± 0.24	182 ± 34	0	0	0	95
24	1.97 ± 0.02	1452 ± 56	8.25 ± 0.45	262 ± 36	2.5 ± 0.32	0	0	85
<i>Silene jaiensis</i>								
12	2.4 ± 0.3	936 ± 19	5.5 ± 0.6	302 ± 23	0	0	0	95
24	2.9 ± 0.04	1002 ± 24	11.25 ± 0.45	248 ± 42	0.63 ± 0.18	0	0	70



2. A slight growth of microshoots, the formation of green leaves and one-three adventitious microshoots per explant were noted.

3. Microshoot thickness in the studied species was 936–1230  $\mu\text{m}$ , *S. jailensis* plants had the thinnest shoots. On the transverse sections of the microshoots, xylem and phloem vessels were visible (Fig. 1); in *S. jailensis*, sclerenchyma was noted.

4. The high capacity for the subsequent development of plants can be estimated by the formed axillary buds (Fig. 1.3).

5. Leaf blades in *L. glaberrimum*, *C. purpurea* and *S. exilis* were bifacial amphistomatic, 159–182  $\mu\text{m}$  thick. *S. jailensis* was characterized by equifacial amphistomatic leaves, 302  $\pm$  23  $\mu\text{m}$  thick. The leaf mesophyll had four-eight cell layers. It was differentiated into palisade and spongy tissue in *L. glaberrimum*, *C. purpurea*, and *S. jailensis*. The epidermis in the leaves was single-layer.

#### Morphometric parameters of explants preserved *in vitro* for 24 months

1. After 24 months deposition under conditions of gene bank *in vitro* at a temperature 4 and 6  $^{\circ}\text{C}$  on MS culture medium supplemented with 60.0 g/L sucrose and 0.2 g/L CCC explants had different viability degree, produced single leaves, adventitious buds, shoots, rosettes, and roots. At the same time, a decrease in the growth kinetics by 1.5–3 times compared to the control was noted.

2. *C. purpurea* regenerants kept high viability for 24 months of non-stop storage (85 %) (Table). We noted slow shoot growth ( $0.98 \pm 0.01$  cm, shoot diameter 980–1200  $\mu\text{m}$ , rosette diameter 1733–2300  $\mu\text{m}$ ), formation of green leaves ( $3.75 \pm 0.16$  ones per shoot, 70–115  $\mu\text{m}$  thick with a homogeneous 5-layer mesophyll), multiple formation of axillary buds, growth and formation of new roots ( $1.75 \pm 0.25$  ones per shoot). The leaves were pedate, and

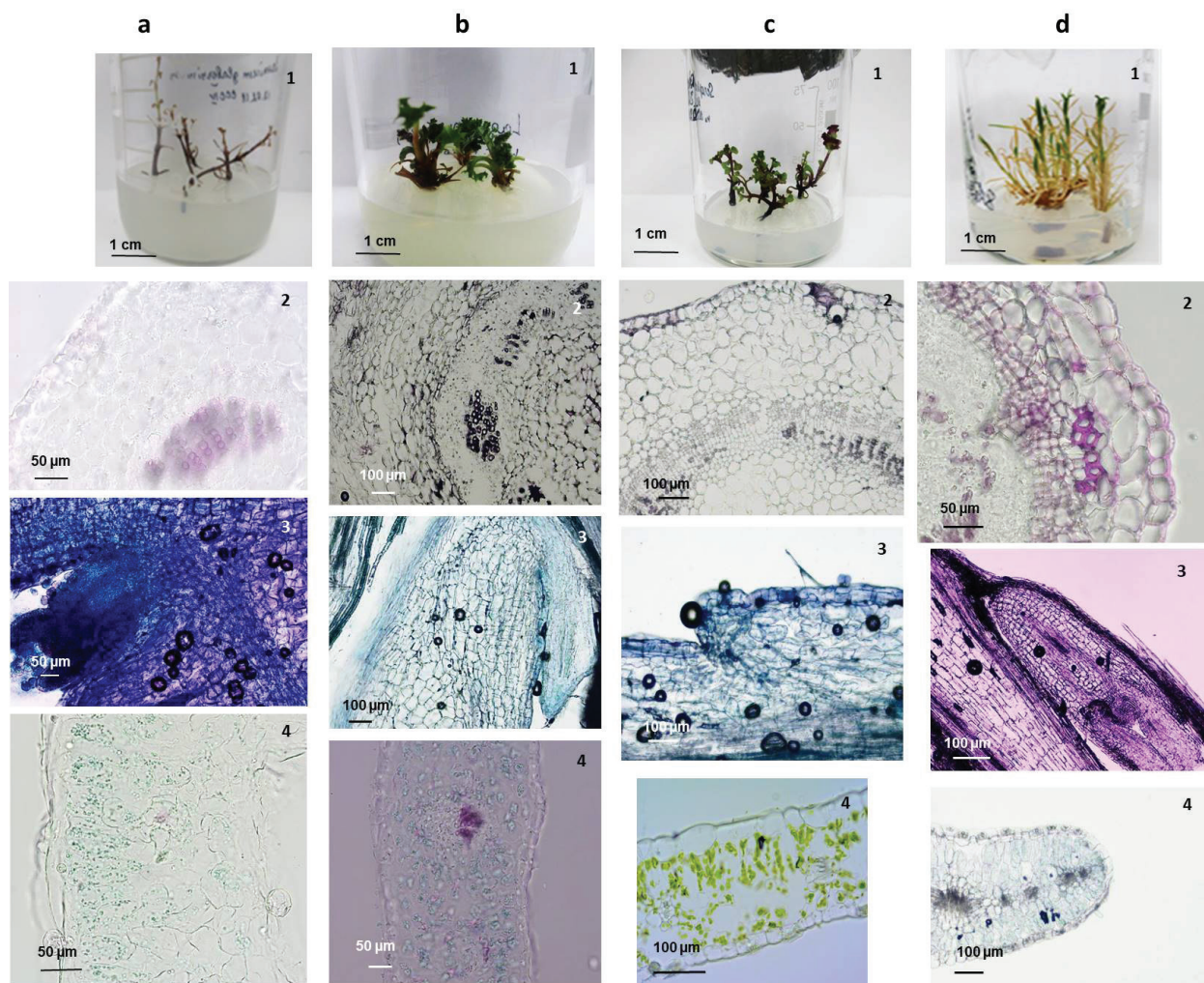


Fig. 1. Endemic species of the Crimean flora under the conservation for 12 months: a – *Lamium glaberrimum*; b – *Crepis purpurea*; c – *Scrophularia exilis*; d – *Silene jailensis*; 1 – general view of plants in the gene bank *in vitro*; 2 – cross section of shoot; 3 – bud on the longitudinal section of shoot; 4 – cross section of leaf.



there were often cases of palmate leaves that were thicker than after 12 months.

3. *L. glaberrimum* explants, on average, formed  $2.38 \pm 0.18$  leaves. Their thickness was the same as after 12 months of *in vitro* deposition. The leaf became a little thicker, large intercellular spaces appeared in the tissues. The newly formed buds developed slowly: leaf primordia were visible (Fig. 2). At the same time, some leaves and shoots had the signs of drying out. The viability was 75 %.

4. *S. exilis* microshoots were  $1.97 \pm 0.02$  cm long,  $1002 \mu\text{m}$  diameter, the number of lateral shoots was  $2.5 \pm 0.32$ , and the total number of leaves was  $8.25 \pm 0.45$ . Under long-term storage, the leaf blades became thinner ( $248 \pm 42 \mu\text{m}$ ) and had an eight-nine layer differentiated mesophyll. The color of

the shoots varied from green to anthocyanin, the leaves – from green to green-brown. The shoots had elongated internodes. All explants had no roots. Explants viability was 85 %.

5. *S. jailensis* microshoots after 24 months of direct culture at a temperature  $4 \text{ }^\circ\text{C}$  had a diameter  $1600\text{--}1800 \mu\text{m}$ , were  $2.9 \pm 0.04$  cm high, retained their viability (70 %), formed adventitious shoots of  $0.63 \pm 0.18$  ones per shoot and  $11.25 \pm 0.45$  leaves per shoot. Green leaves remained only at the top of the shoot. New buds formed in the axils of leaves. It is typical for the development of this species under standard culture conditions on a culture medium with plant growth regulators. Under long-term storage, the leaf blades became thinner ( $258\text{--}312 \mu\text{m}$ ) and had an eight-nine layer mesophyll.

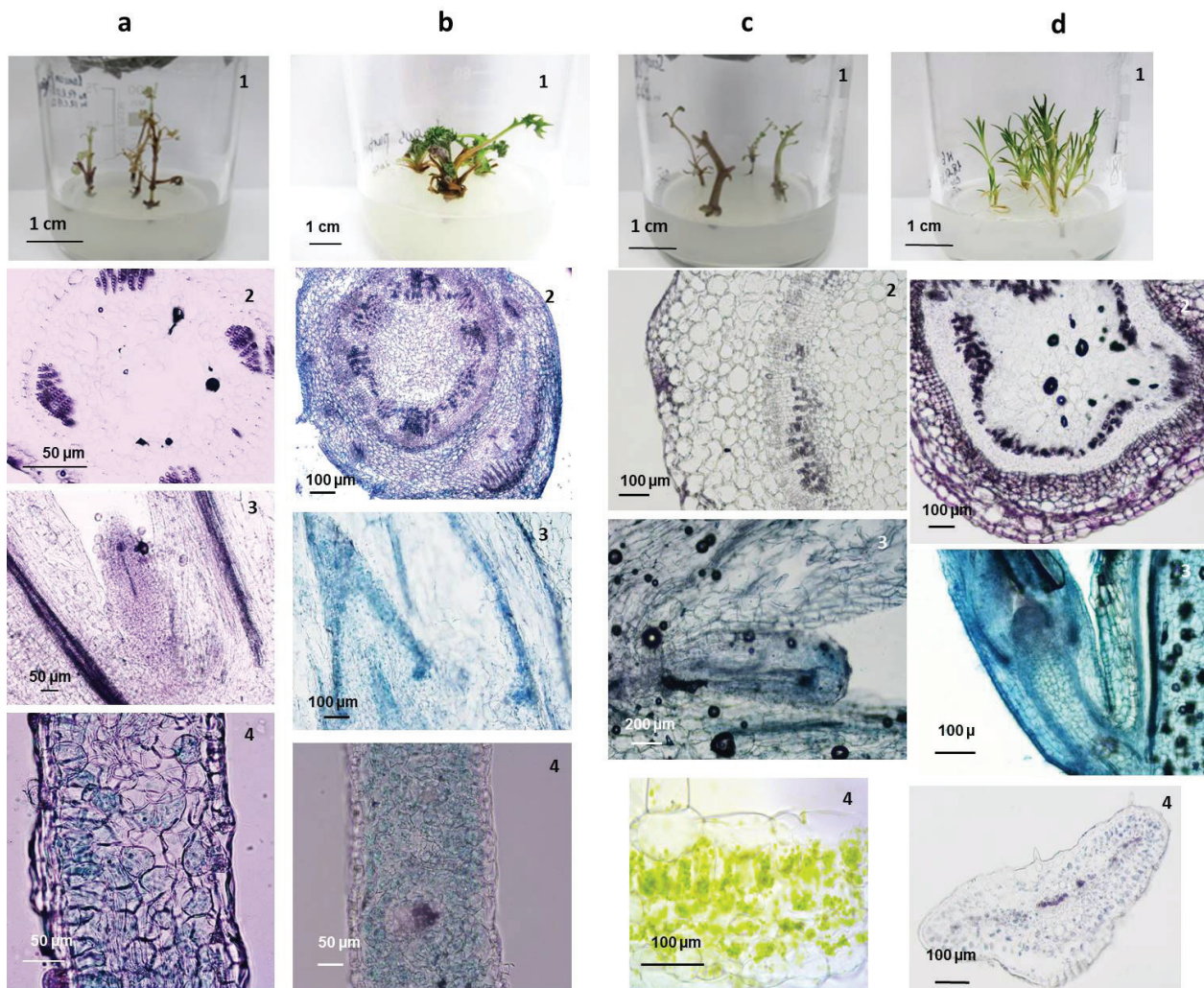


Fig. 2. Endemic species of the Crimean flora under the conservation for 24 months: a – *Lamium glaberrimum*; b – *Crepis purpurea*; c – *Scrophularia exilis*; d – *Silene jailensis*; 1 – general view of plants in the gene bank *in vitro*; 2 – cross section of shoot; 3 – bud on the longitudinal section of shoot; 4 – cross section of leaf.

## Conclusion

The main indicator of the successful conservation of plant species, cultivars and forms is the preservation of their physiological stability (viability) during the entire storage period with a decrease in the growth kinetics. Previous studies made it possible to determine the optimal temperatures (4–6 °C) for conservation of some rare endemics of the Mountainous Crimea. At these temperatures, the explants remained viable with minimal growth (Mitrofanova et al., 2020). The complex use of a number of factors such as sucrose and CCC in the culture medium, optimal temperature and light intensity enabled not only to prolong the deposition time, but also to keep the viability of the studied cultures explants.

Our experiments have demonstrated morphological and anatomical stability in explants of *L. glaberrimum*, *C. purpurea*, *S. exilis*, *S. jailensis* on a culture medium supplemented with osmosis and retardants during 24 months of conservation in the gene bank *in vitro* at temperature – 4 and 6 °C. In all studied plant species, a decrease in viability was noted with an increase in the time of deposition from 12 to 24 months. Along with the retarded shoot growth, the formation of adventitious shoots, leaves,

and roots was observed. These characteristics were noted in a number of scientific works on other cultures (Kovalchuk et al., 2009; Gianni, Sottile, 2015; Rajasekharan, Shijram, 2015; Reed, DeNoma, 2016). The formation of multiple axillary buds may indicate a high regeneration capacity in rare and endemic species. A number of histological features in the leaves of the *in vitro* deposited explants were revealed. These features enable explants to maintain physiological stability when active production processes are stopped. In leaf blades, the thickness and density of the mesophyll decreased, the polysaccharide tissue degraded, and a high rate of transpiration regulation was noted. It was found that under conditions of long-term preservation of explants at an optimal temperature, tissues of regenerants have high viability due to the structural rearrangements of chlorenchyma and keeping a number of characteristics of each plant species under *in vitro* conditions.

## Acknowledgements

The studies were funded by State Assignment of FSFIS “NBG-NSC” and FSFIS MBG RAS and done on the unique scientific installation “FITOBIOGEN”.

## REFERENCES / ЛИТЕРАТУРА

- Bell R. L., Reed B. M.** 2002. *In vitro* tissue culture of pear: Advances in techniques for micropropagation and germplasm preservation. *Proc. 8th IS on Pear.*: 412–418. DOI: 10.17660/ActaHortic.2002.596.66
- Cruz-Cruz C. A., González-Arnao M. T., Engelmann F.** 2013. Biotechnology and Conservation of Plant Biodiversity. *Resources* 2: 73–95. DOI: 10.3390/resources2020073
- Engelmann F.** 2011. Use of biotechnologies for the conservation of plant biodiversity. *In Vitro Cell. Dev. Biol. – Plant* 47: 5–16. DOI: 10.1007/s11627-010-9327-2
- Engelmann F.** 2013. Biotechnology and conservation of plant biodiversity. *Resources* 2: 73–95. DOI: 10.3390/resources2020073
- Fundamentals of creating an in vitro gene bank of species, cultivars and forms of ornamental, aromatic and fruit crops: collective monograph.* 2018. I. V. Mitrofanova (Ed.). Simferopol: “ARIAL”. 260 pp. [In Russian] (*Основы создания генбанка in vitro видов, сортов и форм декоративных, ароматических и плодовых культур: коллективная монография.* Под ред. д. б. н. И. В. Митрофановой. Симферополь: ИТ «АРИАЛ», 2018. 260 с.).
- Gianni S., Sottile F.** 2015. *In vitro* storage of plum germplasm by slow growth. *Hort. Sci.* 42: 61–69. DOI: 10.17221/186/2014-HORTSCI.
- Kovalchuk I., Lyudvikova Y., Volgina V., Reed B.** 2009. Medium, vessels and genotype all influence *in vitro* cold storage apple germplasm. *Plant Cell, Tissue and Organ Culture* 96, 2: 127–136. DOI: 10.1007/s11240-008-9468-8
- Nikiforov A. R.** 2018. Biological and morphological features of some obligate glareophytes of the Mountain Crimea. *Bot. Zhurn.* 103(8): 968–980. [In Russian] (**Никифоров А. Р.** Биологические и морфологические особенности некоторых облигатных гляреофитов Горного Крыма // Бот. журн., 2018. Т. 103, № 8. С. 968–980).
- Nikiforov A. R., Nikiforova A. A.** 2016. Rhythmological differences in the development of *Lagoseris callicephala* and *Lagoseris purpurea* (Asteraceae) plants. *Bull. of the State Nikita Botan. Gard.* 118: 58–63. [In Russian] (**Никифоров А. Р., Никифорова А. А.** Ритмологические различия в развитии растений *Lagoseris callicephala* и *Lagoseris purpurea* (Asteraceae) // Бюллетень ГНБС, 2016. Вып. 118. С. 58–63).
- Novikova T. I.** 2013. Use of biotechnological approaches for plant biodiversity preservation. *Rastitelnyy mir Aziatskoy Rossii [Plant Life of Asian Russia]* 2(12): 119–128. [In Russian] (**Новикова Т. И.** Использование биотехно-

логических подходов для сохранения биоразнообразия растений // Растительный мир Азиатской России, 2013. № 2(12). С. 119–128).

**Mitrofanova I. V., Lesnikova-Sedoshenko N. P., Brailko V. A., Chelombit S. V., Zhdanov, I. V., Mitrofanova O. V.** 2020. Biotechnological methods of propagation for some rare endemic plant species of the southern Russian flora. *Acta Horti* 1285: 177–184. DOI: 10.17660/ActaHortic.2020.1285.28

**Molkanova O., Egorova D., Mitrofanova I.** 2018. Preservation characteristics of valuable plant species in *in vitro* genebanks at Russian botanical gardens. *In Vitro Cell. Dev. Biol. – Plant* 4, 1: S46. DOI: 10.1007/s11627-018-9921-2

**Murashige T., Skoog F.** 1962. A revised medium for rapid growth and bioassays with Tobacco tissue cultures. *Physiol. Plant.* 15, 3: 473–497. DOI: 10.1111/j.1399-3054.1962.tb08052.x

*Red Book of the Russian Federation (plants and fungi)*. 2008. Yu. P. Trutnev et al. (eds); compiled by R. V. Kamelin et al. Moscow: KMK Scientific Press Ltd. 885 pp. [In Russian] (*Красная книга Российской Федерации (растения и грибы)*) / Гл. редколл.: Ю. П. Трутнев и др.; сост. Р. В. Камелин и др. М.: Тов-во науч. изд. КМК, 2008. 885 с.).

**Rajasekharan P. E., Sahijram L.** 2015. *In vitro* conservation of plant germplasm. In: *Plant Biology and Biotechnology. Volume II: Plant Genomics and Biotechnology*. B. Bahadur, M. Venkat Rajam, L. Sahijram, K. Krishnamurthy (eds). New Delhi: Springer. Pp. 417–443. DOI: 10.1007/978-81-322-2283-5\_22

*Red Book of the Republic of the Crimea. Plants, algae and mushrooms*. 2015. A. V. Yena, A. V. Fateryga (eds). Simferopol: “ARIAL”. 480 pp. [In Russian] (*Красная книга Республики Крым. Растения, водоросли и грибы*). Отв. ред. А. В. Ена, А. В. Фатерыга. Симферополь: ООО «ИТ «АРИАЛ», 2015. 480 с.).

**Reed B. M., DeNoma J.** 2016. Medium-term *in vitro* storage of pear as a complementary germplasm preservation technique. *Acta Horti* 1113: 251–256. DOI: 10.17660/ActaHortic.2016.1113.37

**Sarasan V., Cripps R., Ramsay M. M., Atherton C., McMichen M., Prendergast G., Rowntree J. K.** 2006. Conservation *in vitro* of threatened plants – progress in the past decade. *In Vitro Cell. Dev. Biol. – Plant* 42: 206–214. DOI: 10.1079/IVP2006769

**Whitlock R., Hipperson H., Thompson D. B. A., R. K. Butlin R. K., Burke T.** 2016. Consequences of *in-situ* strategies for the conservation of plant genetic diversity. *Biological Conservation* 203: 134–142. DOI: 10.1016/j.biocon.2016.08.006