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CONSERVATION Of SEEDS OF CRIMEAN ENDEMIC SPECIES *ALYSSUM CALYCOCARPUM* RUPR. (*BRASSICACEAE*) AND *ANTHYLLIS BIEBERSTEINIANA* POPL. (*FABACEAE*) IN LIQUID NITROGEN (–196° C)

K e y w o r d s: Alyssum calycocarpum, Anthyllis biebersteiniana, *Crimea*, *endemic species, seeds, liquid nitrogen, cryopreservation, germination*

Abstract
Preservation *ex situ* of the seeds of *Alyssum calycocarpum* Rupr. (*Brassicaceae*) and *Anthyllis biebersteiniana* Popl. (*Fabaceae*), species endemic to the Crimean flora, has been studied using modern cryotechnique. The seeds survived after having been exposed to ultra low temperature (–196° C) during a month.

Introduction

The importance of the preservation of seeds as germplasm resources of the world flora has been emphasized already by N.I. Vavilov. Black in his report [15] later noted that large amounts of experimental data were generated in the field of seed biology during the recent years and, as a result, have changed our understanding of the role of seeds in plant conservation. According to current scientific data, seeds (in particular, their DNA) contain all genetic information of the genotype formed during the process of species evolution. Because of that seed (sexual) reproduction provides the inheritance of the whole spectrum of species' genetic variability as compared to vegetative (asexual) reproduction. According to international standards, an integral part of the general principle of plant conservation is preservation of seeds in *ex situ* conditions, i.e. in special seed depositories [12]. The conservation of plant diversity primarily implies wild species conservation. The aim of the Millennium Seed Bank project by 2020 is to save 25 % of species of world's wild plants with bankable seeds [18].

In Ukraine, the seeds of wild and introduced plants are collected and preserved mainly at botanical gardens. As a rule, seeds preservation and storage occurs under the room temperature. These conditions causes the decrease or loss of the germination ability of seeds. The fundamental investigations, including those by Navashin [10], emphasized that preservation under these conditions has a negative impact on quality of certain seeds and leads to their germination decrease. Experiments have shown that the best method of long-term storage of plant material to ensure genetic stability within a long period of time is cryopreservation, e.g. conservation in liquid nitrogen under –196° C. This method is considered to be an important and up-to-date tool for plant conservation, especially for the seed material that forms national natural resources. Cryopreservation has several important advantages as compared to other *ex situ* conservation techniques [16, 20]. It provides a possibility to protect plant material against the influence of main adverse abiotic and biotic factors. The first studies in this field in Ukraine were conducted by Grishenkova for six Carpathian species [2]. During the past few years, plant cryopreservation protocols were developed at the Botanical Garden of Ivan Franko National University of Lviv [14]. The research mostly concerned rare and endemic species of the Ukrainian flora.

Material and Methods

The reported research covers selected rare and narrow-range taxa of the Crimean native flora. Although the Crimean Peninsula occupies only 4.2 % of the territory of Ukraine, it is one of the European hotspots of biodiversity. The Crimea is considered to be the richest floristic region of the country. It has the largest concentration of endemic species (from ca. 120 to 300, according to various assessments) [4, 5, 19]. The taxa investigated in the present study were *Alyssum calycocarpum* Rupr. (*Brassicaceae*) and *Anthyllis biebersteiniana* Popl. (*Fabaceae*) that were introduced in the University Botanical Garden. The species are perennial plants, and the data of their prior introduction were absent [7, 11].

Alyssum calycocarpum is believed to be a species restricted to the Crimean-Novorossiysk region [5]. This species has the «R» status for the European part of the former USSR in the IUCN Red List [17], i.e. it is under

the threat of extinction. *A. calycocarpum* predominantly grows on limestone and dry slopes of southern beaches [9]. Iljinska [6] refers to Ruprecht who named the species, found *A. calycocarpum* in many places of the Crimea, and described its location in the Mountain Crimea and the Novorossiysk floristic region of the Caucasus. *A. calycocarpum* is an ornamental plant. Its shoots are simple or branching, up to 35 cm tall, petals are yellow, and the silicles are oval or globular. Its flowering usually starts in May—June [9]. Under the introduction conditions, flowering starts in late May and full flowering — at the beginning of June. Seeds ripen in July.

Anthyllis biebersteiniana is a narrowly endemic species connected with the alpine elements of the flora of the Crimean mountain yailas (treeless mountain tops) [13]. According to Cherepanov, the species also occurs in the Caucasus [cit. by 5]. It grows on rocky areas of the mountain steppes of the Crimea [13] and on meadow steppes of yailas [9]. The flowering of *A. biebersteiniana* occurs in June—July under botanical garden conditions, and seeds ripen in August—September. Under the introduction conditions, the species produces vital seeds that have no resting period, as for the previous species. An average indicator of laboratory germination of the freshly collected seeds of *A. biebersteiniana* is 46.7 %, and the germination ability under the room conditions decreases every month [8], equaling 12 % after the period of 6 months.

Cryopreservation of plant material was conducted in the Cryoconservation Scientific and Technical Centre of Low Temperature Studies at Ivan Franko National University of Lviv. The collected seeds of experimental species that had been kept for several months in laboratory conditions were then transferred to the plastic Eppendorf tubes (50 seeds in a threefold repetition for each species) and were dipped directly into liquid nitrogen for one month of conservation under the temperature of −196° C. After the experiment, the seeds were warmed under laboratory conditions. Slow warming was performed under the room temperature by leaving seeds in the Eppendorf tubes for 3 days. The control seeds were kept under laboratory conditions. The seeds were then sown and grown in soil boxes. The survival rate was estimated as the percentage of seeds that germinated after freezing and thawing. The significance of differences between various treatments was evaluated by special software [3]. The Wellington method [1] was used for the detection of seedlings growth and development.

Table 1. **Soil germination dynamics of *Alyssum calycocarpum* seeds after cryostorage**

Day of germination	Control		Frozen for 4 weeks	
	Quantity of germinating seeds	Percentage of germinating seeds	Quantity of germinating seeds	Percentage of germinating seeds
8th	4.3±1.3	15.5±4.8	6.7±3.8	13.3±7.7
14th	6.0±1.7	21.4±6.2	9.0±4.6	18.0±9.2
16th	6.0±1.7	21.4±6.2	9.3±3.9	18.7±7.9

Results

Seeds of *Alyssum calycocarpum* under investigation preserved their viability after their conservation in liquid nitrogen for a month. The control seeds (unfrozen ones) and experimental variants were germinated for two weeks (Table 1).

The final percentage of seed viability in the control variant (21.4±6.2) was higher than that for the frozen seeds (18.7±7.9). As the number of investigated samples was great (n=150), the unreliability of differences confirms that the germinating ability of the frozen seeds did not change as compared to the unfrozen ones. The opening of cotyledons was registered on the 14th day of the germination beginning both in the control and treatment variants. The first leaves appeared on the 20th day of germination. Storage of seeds in liquid nitrogen did not affect the growth and development of the cryopreserved diaspores. The seeds stored at −196°C had the same appearance and growth patterns as the unfrozen (control) ones. Seedlings were growing normally showing the same morphology as the plants recovered from the unfrozen control seeds. The increase in size and color intensification of leaves was registered for both variants.

For *Anthyllis biebersteiniana*, the same strategy of seed germination was applied. Both in control and experimental variants, the seed germination in the was registered on the 4th day after sowing. However, the period of seed germination in the control variant was more prolonged than that of the frozen variant (Table 2).

Table 2. **Soil germination dynamics of *Anthyllis biebersteiniana* seeds after cryostorage**

Day of germination	Control		Frozen for 4 weeks	
	Quantity of germinating seeds	Percentage of germinating seeds	Quantity of germinating seeds	Percentage of germinating seeds
4th	1.3±0.9	2.7±1.8	1.7±1.2	3.3±2.4
11th	1.3±0.9	2.7±1.8	2.7±1.5	5.3±2.8
14th	3.3±1.3	9.3±1.3	5.7±2.0	11.3±4.1
18th	4.7±1.8	9.8±3.5	-	-

Table 3. **Survival of seeds after cryopreservation during one month**

Object	Percentage of germinating seeds	
	Control	Frozen
Alyssum calycocarpum	21.4±6.2	18.7±7.9
Anthyllis biebersteiniana	9.8±3.5	11.3±4.1

The cotyledons appeared earlier in control seedlings than in the frozen ones. The first leaves appeared three weeks after sowing, simultaneously in the unfrozen and frozen variants. However, the seedlings of the seeds treated by low temperatures were delayed in their development as compared to the control ones, and were 1—1.5 cm tall while the control ones were 2—3 cm tall.

The seeds of both investigated species, *A. calycocarpum* and *A. biebersteiniana*, preserved their germinating capacity (Table 3). The germination of freezing/thawing treated seeds started at the same time as the germination of non-treated ones. The seedlings were developing normally and had the same morphology as those from the unfrozen control group. All the seedlings produced green leaves. There were seedlings with well-formed leaves.

The difference between the percentage of seeds germination in the control and experimental groups is not statistically reliable. Thus, seed germination in the experimental variant changes insignificantly as compared with the control one.

Conclusion

We report the successful cryopreservation of *Alyssum calycocarpum* and *Anthyllis biebersteiniana* seeds. These results showed that ultra low temperature (–196° C) did not negatively influence the seed germination and seedlings growth of seeds of *A. calycocarpum* and *A. biebersteiniana*.

As shown in this study, conservation of plant material using liquid nitrogen is consistent with the requirements of durable preservation of plant genetic information without negative changes of their gene structure and biological characteristics. The method is environment-friendly and cost-effective. The main practical problem in cryopreserving plant material is the selection of freezing and thawing conditions for each plant species. Our analysis of the experimental data suggests that seeds of introduced species *A. calycocarpum* and *A. biebersteiniana* are suitable for conservation in liquid nitrogen by means of direct immersion. The investigated seeds preserve their viability without the use of cryoprotective substances. The cryopreservation protocols were developed for the seeds of these two taxa.

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ЗБЕРІГАННЯ НАСІННЯ ЕНДЕМІЧНИХ ВИДІВ ФЛОРИ КРИМУ *ALYSSUM CALYCOCARPUM* RUPR. (*BRASSICACEAE*) ТА *ANTHYLLIS BIEBERSTEINIANA* POPL. (*FABACEAE*) У РІДКОМУ АЗОТІ (–196° С)

Зберігання *ex situ* насіння *Alyssum calycocarpum* Rupr. (*Brassicaceae*) і *Anthyllis biebersteiniana* Popl. (*Fabaceae*), ендемічних видів флори Криму, досліджували з використанням сучасного методу кріотехнології. Насіння не втратило життєздатності після перебування його протягом місяця у рідкому азоті.

Ключові слова: Alyssum calycocarpum, Anthyllis biebersteiniana, *Крим, ендемічні види, насіння, рідкий азот, кріозберігання, схожість.*

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СОХРАНЕНИЕ СЕМЯН ЭНДЕМИЧЕСКИХ ВИДОВ ФЛОРЫ КРЫМА *ALYSSUM CALYCOCARPUM* RUPR. (*BRASSICACEAE*) И *ANTHYLLIS BIEBERSTEINIANA* POPL. (*FABACEAE*) В ЖИДКОМ АЗОТЕ (–196° С)

Хранение *ex situ* семян *Alyssum calycocarpum* Rupr. (*Brassicaceae*) і *Anthyllis biebersteiniana* Popl. (*Fabaceae*), эндемических видов флоры Крыма, исследовали с использованием современной криотехнологии. Семена сохранили жизнеспособность после хранения их в течении месяца в жидком азоте.

Ключевые слова: Alyssum calycocarpum, Anthyllis biebersteiniana, *Крым, эндемические виды, семена, жидкий азот, криосохранение, всхожесть.*

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