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# *IN VITRO* CULTIVATION OF RARE AND ENDEMIC *ALLIUM* SP. SPECIES

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## ABSTRACT

**Here present the results of studies on the *in vitro* cultivation of rare, dangerous and endemic species of *Allium* genus (*A. ledebourianum, A. altaicum, A. schoenoprasum, A. obliquum*) grown in the territory of the Kazakh Altai. The seeds of onions collected in the locations of their natural growth, namely the territory of the Kazakh Altai, were used as material to conduct the study. The sterilization protocols for seeds of various *Allium* species were established. It was found that it is necessary to apply the procedure based on multiple-step disinfection and various sterilizing agents to obtain aseptic shoots of different *Allium* species. The seed germinating efficiency of rare and endemic species of *Allium sp. in vitro* conditions were analyzed during the study.**

**Key words: endemic, *in vitro* culture, *Allium*, population, stratification, phytohormone.**

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## INTRODUCTION

The protection of rare, endangered, and endemic plant species is one of the aspects of conservation of biological diversity. More than 50,000 species of wild plants are used throughout the world as sources of pharmacological substances, food products, and in perfumery. Such species often and steadily tend to reduce populations, loss of biodiversity until complete disappearance. Approximately 50% of the endemic species that provided up to 16% coverage in the foreseeable past have now been reduced to 2.3% of the earth's surface [1]. This indicates an unprecedented decline in the world biodiversity of endemic flora. In Kazakhstan, about 14% of the flora or about 700 species belong to rare and endemic plant species, which are the most vulnerable, because they have small natural reserves and narrow habitats [2].

Species of the genus *Allium* L. are perennial herbaceous plants belonging to the subfamily *Alliaceae*, and family *Amaryllidaceae*. Most species of this genus are perennials with bulbs or rhizomes that have a sharp specific flavour and taste. Virtually all *Allium* species are recognized as valuable food, medicinal or ornamental plants that long-harvested or extracted by a man since ancient times, which leads to a significant depletion of their stocks in nature.

This genus includes more than 900 species, most of which are widely grown in the Northern Hemisphere, as well as distributed and diversified in the steppe, desert or

semi-desert zones [3]. In addition, mountainous territories of Kazakhstan are proposed as centre of the variability of *Allium* species, which is confirmed by a large number of its endemic, relict and rare species in the Altai and Tien Shan [4]. The Kazakh flora is endowed with a rich diversity of this genus, which includes over than 140 species, of them 45 are endemic [5]. Many species of *Allium* especially decorative, medicinal and food have become rare or disappearing and now need protection. A significant scientific interest in studying is florogenesis of rare Sayan-Altai and narrowly grown endemic species of *Allium* species (*A. altaicum* Pall., *A. ledebourianum* Schult. et Schult. Fil.), as well as long-root onion (*A. microdictyon Prokh.*) that grown on the southwestern boundary of the Altai Mountains [6].

Such species as *A. ledebourianum, A. altaicum, A. schoenoprasum L., A. obliquum L.* are the glacial relict plants, which reduce their numbers throughout the world due to mass harvesting for food purposes. In connection with the change in the habitat under the influence of man's economic activity, the natural location of relict plants is reduced. The abusive mass harvesting destroys mother plants, which significantly reduces the seed renewal of these species and their habitats and distribution areas. Currently, these species are found only on hard-to-reach slopes, populations are sporadic, and some include only some species [6].

Rare and endemic species of onion such as *A. ledebourianum*, *A. altaicum*, and *A. microdictyon* are included in the Russian Regional Red Lists and are subject to state protection [7-11]. Despite the fact that these species are included in the List of Species Subject to Prohibitions or Restrictions on Import or Export within the Eurasian Economic Community [12], in Kazakhstan they are still not specifically protected [13].

Preservation of the biological diversity of rare and endemic plant species is one of the most important tasks of nature protection, which is given great attention around the world, including Kazakhstan that ratified the "Convention on Biological Diversity" in 1995.

In this regard, *in vitro* technologies are considered as one of the necessary options in the biodiversity conservation strategy, since they allow not only to obtain a sufficient amount of valuable material free of infections, but also to save space and labour resources aimed at maintaining culture collections [14]. *In vitro* conservation technologies are used to preserve rare wild species that exist in hard-to-reach areas, have reduced seed production or developmental disabilities, or when populations are represented by only a few individuals [15].

Unlike traditional methods of sample conservation, *in vitro* technology is a multi-stage process consisting of the collection of material, cultivation *in vitro*, micropropagation, assessment of genetic purity and maintenance of samples under slow growth conditions or their cryopreservation.

The initial stage of biotechnology used for conserving flora biodiversity is to introduce plants into the culture *in vitro*. For most wild species, this stage is complicated by the fact that all parts of such plants are exposed to pathogens to varying degrees. Contamination of both exogenous and endogenous nature is the main obstacle for *in vitro* culture of plants.

The level of contamination is affected by various factors including the plant age, tissue specificity and environmental conditions. In this regard, surface sterilization is the first and most important step to obtain aseptic cultures. This stage is especially important for endangered species, mainly wild endemic. In this connection, the use of seeds as primary explants is most preferable, since it allows obtaining a fairly representative genetic collection of the species, with the use of rigid sterilization schemes and highly toxic antiseptics to reduce surface contamination [16].

Surface sterilization by applying various antiseptics such as ethanol, potassium permanganate, and hydrogen peroxide is sufficient against pathogenic microorganisms

of exogenous nature. According to Sarasan V., et al (2006), sodium dichloroisocyanurate (SDIC) is one of the optimal antiseptics for plant explants with minimal phytotoxicity [16]. In the case of endogenous contamination, surface sterilization will not be sufficient, it is necessary to use multi-stage protocols to sterilization by applying several different antiseptics. In addition, antibiotics and fungicides can be introduced in a medium to prevent bacterial or fungal infection.

Thus, the cultivation *in vitro* is the first and most complex stage in the technology for conserving plant biodiversity. Therefore, the conducted studies were devoted to optimizing the stages for obtaining viable aseptic cultures of rare and endemic *Allium sp*. species.

## MATERIALS AND METHODS

Seeds of various *Allium* species including *A. ledebourianum, A. altaicum, A. schoenoprasum, A.obliquum* collected in places of their natural growth in the territory of the Kazakh Altai were used as materials for conducting the study.

Surface sterilization of explants was carried out using various antiseptics, including ethanol, hypochlorite, mercuric chloride, potassium permanganate, and hydrogen peroxide [17, 18]. The following protocols were used:

Protocol No. 1: ethanol 70% – 40 seconds, mercury chloride 0.1% – 20 minutes.

Protocol No. 2: hydrogen peroxide 3% – 10 min., mercury chloride 0.1% – 20 min.

Protocol No. 3: potassium permanganate 2% – 20 min., mercury chloride 0.1% – 20 min.

Protocol No. 4: sodium hypochlorite 10% – 15 minutes.

Protocol No. 5: ethanol 70% – 30 seconds, sodium hypochlorite 5% – 20 minutes.

Protocol No. 6: ethanol 70% – 30 seconds, sodium hypochlorite 10% – 20 minutes.

Protocol No. 7: ethanol 70% – 30 seconds, sodium hypochlorite 15% – 20 minutes.

Protocol No. 8: ethanol 70% – 30 sec., mercury chloride 0.1% – 5 min., sodium hypochlorite 20% – 20 min.

After each antiseptic treatment, the seeds were washed with distilled water.

The *in vitro* cultivation of plants was carried out after preliminary sterilization. Seeds were placed in nutrient media containing 3-indole acetic acid (IAA) and gibberellic acid (GA3) various concentrations. The composition of all nutrient media included sucrose (30 mg/l) and agar (6-8 mg/l).

## RESULTS AND DISCUSSION

*Obtaining a collection of in vitro culture of onion seeds*

To obtain a collection of endemic *Allium* species, officials from the Altai Botanical Garden provided seeds harvested in places of natural growth in the territory of the Kazakh Altai (fig. 1).

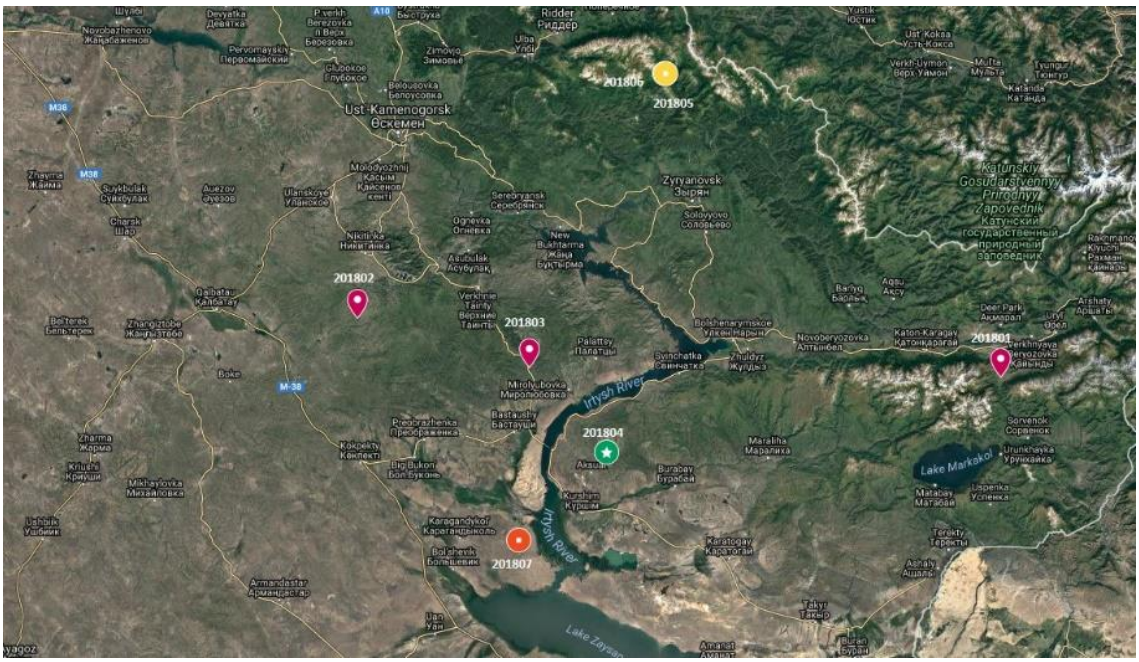


**Fig. 1.** The places of a collection of samples of rare and endemic species of alliums in the territory of Kazakhstan's Altai

The limiting factors for the wide distribution of these species are their weak ability to form daughter bulbs and low seed production, which is caused by environmental stressors such as dry hot summers and cold snowy winter [19]. In natural habitats seeds grow over long periods and even a few years [20]. The productivity is also affected by a dense vegetation cover that does not allow vegetative reproduction and massive abusive gathering by population. The collection includes the following species of alliums (table 1).

**Table 1.** Collection of rare and endemic species of alliums

| ID sample | Kind | Place collected | Locations | Height m a.s.l. |
|---|---|---|---|---|
| 201801 | *A. altaicum* | EKR, Katon-Karagay region, Southern Altai, the ridge of South Altai Tarbagatai, in the area of the Burhat Pass. | N-49°07’33’’ E-86°02’10’’ | 2146 |
| 201802 | *A. altaicum* | Kalbinsky Altai, the Kalbinsky Range (Eastern Kalba), the Koktau Mountains. | N-49°29’37’’ E-82°36’19’’ | 693 |
| 201803 | *A. altaicum* | EKR, South-Western Altai | N-49°07’33’’ E-83°32’44’’ | 774 |
| 201804 | *A. schoenoprasum* | South Altai, Katon-Karagay region, Narym ridge, Kainar village. | N-48°49’46’ E-83°48’08’’ | 834 |
| 2018005 | *A. schoenoprasum* | EKR, the southern part of the Lineiskii range, Barsuk river | N-50°19’20’’ E-84°11’26’’ | 1977 |
| 201806 | *A. ledebouriamum* | EKR, the southern part of the Lineiskii range, Barsuk river, waterlogged meadows | N-50°19’12’’ E-84°11’49’’ | 1925 |
| 2018007 | *A. obliquum* | South Altai, Narym ridge, northeastern gravelly slope. | N-48°46’31’’ E-83°28’22’’ | 450 |

The rhizome and bulblet-production species *Allium schoenoprasum L.* (chives) have more economic value. This circumpolar species is widely distributed on the

northeast of European Russia, namely on pebble areas, rocky river banks, floodplain and grassed meadows, tundra communities, limestone outcrops in river valleys, and occasionally in swamps [21]. This species is of interest primarily for breeding, as a source of genes resistant to powdery mildew, and it is quite often and successfully used to obtain interspecies hybrids [22]. The collection includes 2 populations of this species.

Relict and short-rhizome species *Allium altaicum* listed in the Red Lists of Russia, Mongolia and China, is presented in the form of small natural populations that have sporadic (single) distribution on stony slopes of the South-Western Altai [ 23]. The collection includes 3 populations.

*Allium ledebourianum* of the rhizome and bulblet-production type is an endemic narrow-distributed in the territory of the Western Altai.

*Allium obliquum* is the perennial rhizome-bulblet production plant and a species of the Pleistocene floristic complex. This ephemeroides plant producing only 1 bulb, species included in the Regional Red Books of Russia, Ukraine, and Mongolia [24].

***Optimizing the protocol for sterilization of seeds of rare and endangered species of onion in vitro***

Obtaining sterile shoots *in vitro* is the initial stage for subsequent IVC (*in vitro collecting*) technologies: microclonal multiplication, induction of somatic embryogenesis, and callus culture.

The process to obtain collections *in vitro* is complicated due to the fact that donor plants in the natural environment are inevitably exposed to contamination by fungal and bacterial microflora. Endophytic microorganisms, constantly existing on the surface of plants, are a kind of obstacle to obtaining sterile cultures under *in vitro* conditions, because their food needs are the same as in plants.

When cultivated in a nutrient medium microorganism compete with plants for food sources, they can produce various phytotoxins that inhibit the growth of plant tissues. In this regard, the production of sterile shoots free of bacterial and fungal infections is a critical factor affecting the success of IVC technology (*in vitro collecting*) [25].

The main objective of this stage was to obtain non-contamination seedlings of endemic *Allium* species while maintaining the high viability of explants. For this purpose, we used 8 sterilization protocols for all *Allium* species. The surface-sterilized seeds were placed in the Murashige and Skoog medium containing ½ mineral salts, 5 mg/l gibberellic acid (GA3), 0.1 mg/l indole-3-acetic acid (IAA), and allowed to cultivate at 25°C and 16-hour lighting. Gibberellin and IAA are the most important endogenous phytohormones, which are often used both to stop seed dormancy of many species, and to accelerate the germination of seeds of plant species that have no dormancy [26].

In standard mediums for the cultivation of plant tissues, many bacteria or fungi are visible for three or four days, and some can be seen 24 hours after cultivation. Due to the fact that some microorganisms can be slow growing, not appearing for several weeks, the effectiveness of sterilization was assessed after 25 days. After the expiration of this cultivation period, no new infected sprouts appeared which indicated the absence of latent infection and allowed to evaluate the effectiveness of the sterilization protocol (fig. 2).

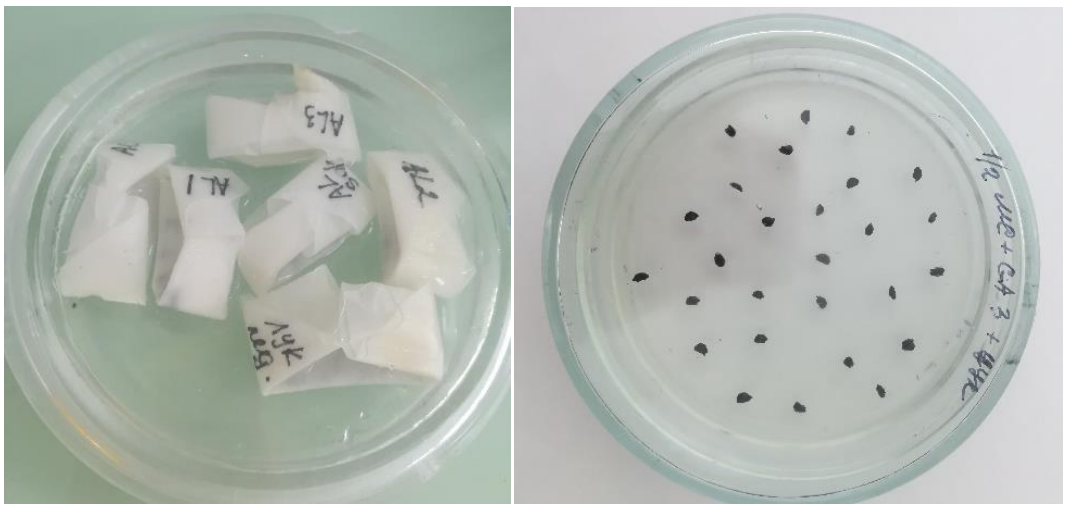

**Fig. 2.** Development of a protocol for the sterilization of seeds of alliums plants *in vitro*

Criteria for choosing the most effective protocol were the number of infected explants (%), and the number of viable shoots (%).

The results showed that the level of surface contamination is determined by the type of antiseptics used, the duration of their exposure, and the combined effect of sterile agents. Exposure of 0.1% mercuric chloride solution in combination with ethanol, hydrogen peroxide and potassium permanganate was disastrous not only for the surface microflora but also for the shoots themselves, which affected their viability. Despite the high efficiency of mercury chloride as a disinfectant of plants, our studies have found high toxicity of this compound for onion shoots, which indicates the need for careful selection of concentration and exposure time to reduce damage to plant tissues. Sodium hypochlorite in concentrations of 10-20% was more effective against pathogenic microflora, which contaminated the seeds and at the same time was less toxic for shoots. The use of sodium hypochlorite at a low concentration of 5% was ineffective (protocol No. 5), since it was not possible to achieve a sterilizing effect on the microflora, all seeds in this variant were contaminated with pathogenic microorganisms (fig. 3).

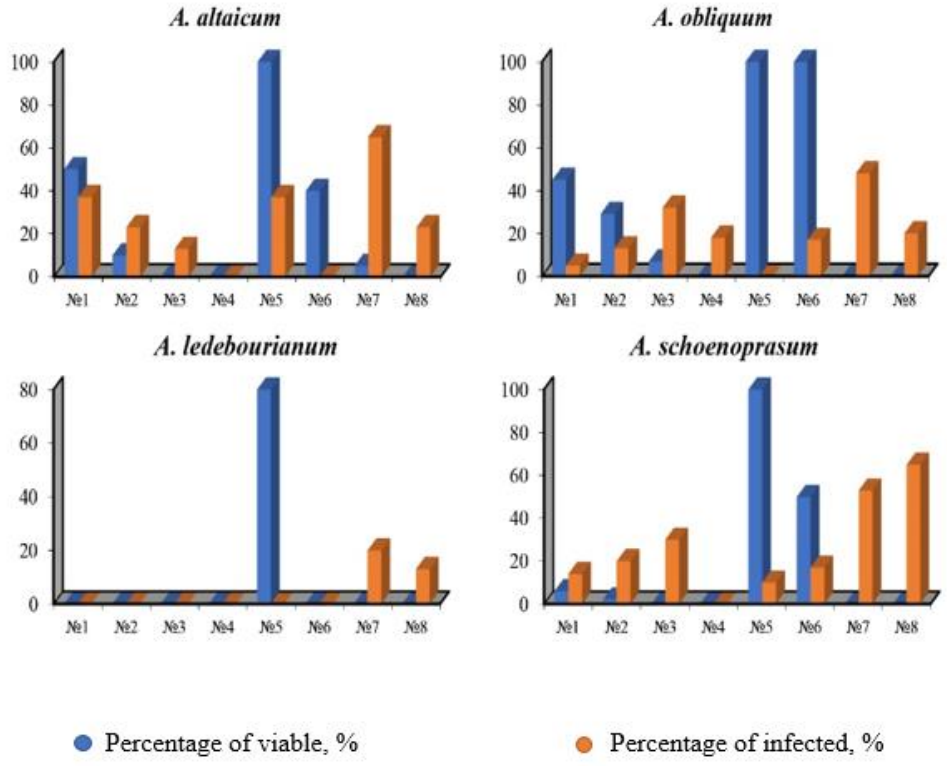


**Fig. 3.** The choice of the optimal protocol for the sterilization of seeds of rare and endemic species of alliums

The reaction specificity to antiseptics of various *Allium* species used was manifested in the intensity of seed germination, which may be due not only to the toxicity of the disinfectants used but also to features of species. So, it was very difficult to get sterile shoots of onion *A. ledebourianum*, the highest number of viable seedlings was obtained using two-stage sterilization protocols based on ethanol and sodium hypochlorite.

Thus, as a result, the protocols for obtaining *in vitro* sterile seedlings of rare and endemic *Allium* species were optimized. It was shown that in case of step sterilization or disinfection with the use of different types of antiseptics, the probability of development of infection within the seedling in a latent form is reduced.

***The efficiency of germinating of seeds of rare and endemic species of onions in vitro conditions***

Researches on the germination of seeds of rare and endemic species are relevant both for solving propagation problems and for conservation using *in vitro* technologies. The seeds of many onions have an underdeveloped fetus occupying less than ¼ of the seed volume, and a copious dense endosperm. In addition, the seeds of bulbous plants are characterized by a state of deep dormancy, therefore, immediately after ripening, even under favourable conditions for a given species, they are unable to germinate or have a decreased germination capacity. In natural habitats the germination of seeds is stretched and can take even a few years [22]. The rate of germination is determined by the level of soil moisture, availability of oxygen and maintenance of optimal temperature. The diversity during the maturation of seeds and their ability to fall into a state of dormancy developed in plants during evolution as a protective reaction to survive the unfavourable periods of the year [27].

To overcome the dormant period, various methods of stratification (cold and/or heat), scarification (mechanical or chemical), exposure to light and humidity are often used, but this takes quite a long time, and in regards of wild species, these techniques are low effective. Biotechnology offers new advantages for overcoming the period of seed dormancy by using endogenous phytohormones in an *in vitro* culture. Phytohormones are the main factors to regulate ontogeny, including germination of seeds.

Gibberellic acid, which stimulates shoot growth due to cellular stretching and an increase in the number of mitoses, and indolyl-3-acetic acid, IAA, with the direct participation of which there is a loosening of the structure of the cell walls and their plastic stretching after an increase in the volume of the vacuole, are most often added to the culture media used to produce the embryo culture. Auxins together with cytokinins activate division of (first) and stretching (second) of cells, as well as provide interaction of parts of the embryo [28].

The studies have revealed that during the swelling period seeds have no necessary balance of phytohormones, their necessary concentrations are established already in the process of germination. In this regard, exogenous phytohormones will significantly increase the germination capacity of swollen seeds, in comparison with dry ones [29, 30]. The use of phytohormones does not replace stratification processes, and greatly reduces the duration of the seed germination.

In our experiments, sterile seeds of *Allium* species were placed in Petri dishes on the nutrient ½MS medium solidified with growth regulators – gibberellic acid and indole-3-acetic acid, 10 ml/l and 0.1 ml/l, respectively, and allowed to cultivate at 25°C with 16-hour lighting. Under such conditions, the seeds of various *Allium* species began to germinate a few days after *in vitro* cultivation. Germination of the seed began with appearing of the root tip through the seed coat. Then the lower part of the seed lobe appeared, it became green and photosynthesized, while the upper part remained in the seed.

The results showed that the germination period varied significantly. Thus, the seeds of *A. obliquum* and *A. schoenoprasum* species, which already had individual shoots on the third day, grew most rapidly, and after two weeks germination reached 40%. The seeds of *A. altaicum* germinated 1-2 weeks after their placing on the nutrient medium, but the species is distinguished by the fastest formation of the micro-bulb *in vitro* conditions (fig. 4).

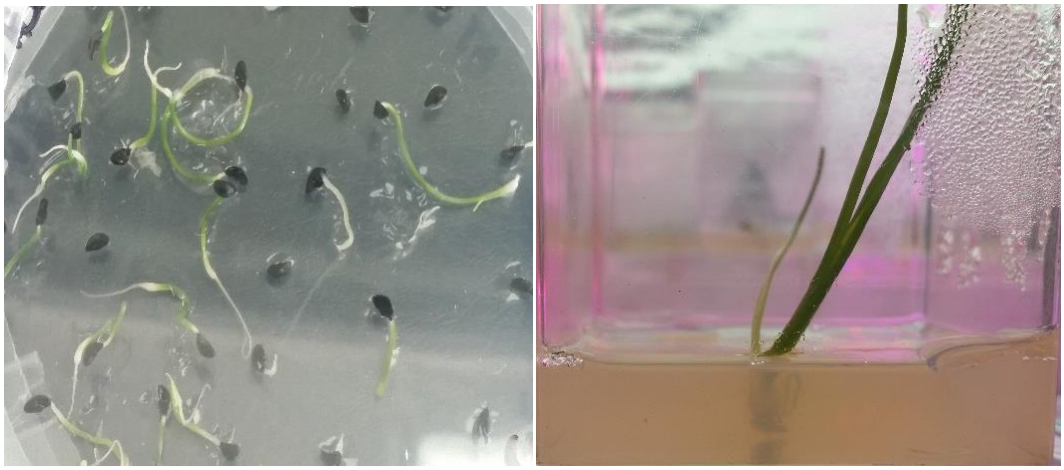

**Fig. 4.** Cultivation of *A. altaicum in vitro*

It was found that the seeds *A. ledebouriamum* have the longest germination period – 4-6 weeks after placing on a nutrient medium. While observing the developing shoots of the *A. ledebouriamum*, we noted that shoots have anthocyanin colour, which is absent in other *Allium* species, which is probably a biological feature of this species (figure 5).

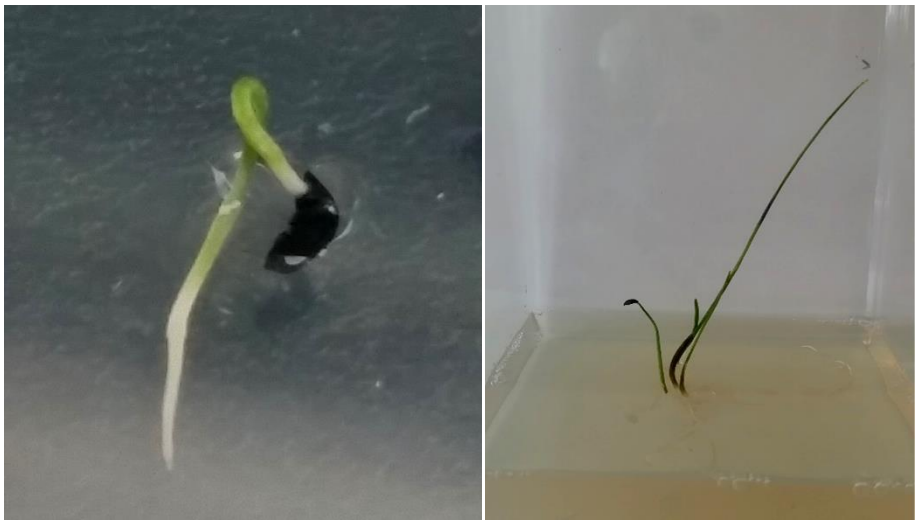

**Fig. 5.** Germination and development of *A. ledebouriamum in vitro*

According to Khlestkina E.K. (2013), the manifestation of anthocyanin colour may indicate the photoprotective action of a pigment, which protects various cellular structures from destruction under the influence of stressful environmental conditions. In addition, some flavonoids have antimicrobial properties [31].

The revealed differences in the germination period of seeds of the investigated *Allium* species indicate the species-specificity on the individual stages of ontogenesis, which are manifested in the presence of adaptive changes in the period of deep dormancy.

Thus, the studies optimized individual stages of cultivation *in vitro* conditions of various Allium species: seed sterilization, obtaining of sterile shoots. Studies have shown the possibility of practical use of the advantages of *in vitro* culture to overcome the period of dormancy of seeds.

## CONCLUSION

The problem of conservation and protection of rare endangered plant species is now becoming urgent due to irrational use of natural resources, an increase of human impact on the environment, deterioration of the ecological situation. Many species, due to a reduction in their numbers and distribution, are threatened with disappearance. In this connection, it is necessary to protect and renew natural populations of the endemic plant. Different *Allium* species are rare and endemic, populations of which are largely reduced in recent times due to grazing and abusive harvesting by the population. The application of biotechnology methods will allow a more complete and rational approach to the use of resources of various *Allium* species both for enriching the natural flora of the Kazakh Altai and for the conservation of rare endangered species.

The studies formed a collection of seeds of *Allium* species such as A. *ledebourianum, A. altaicum, A. schoenoprasum, and A. obliquum*. The collection consists of seven populations of alliums collected in places of natural growth in the territory of the Kazakh Altai. The most effective to culture *in vitro* is the two-stage sterilization in 70% ethanol (exposure time 30 sec.), 20% sodium hypochlorite

(exposure time 20 min.) and 0.1% mercuric chloride (exposure time 5 min), with the optimum ratio of sterility and viability of the seedling. The germination period of seeds is determined by specific features: the seeds of *A. obliquum* and *A. schoenoprasum* germinated on the third day, *A. ledebouriamum* seeds have the longest germination period – 4-6 weeks after placing on the nutrient medium.

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