Growing cell cultures of some rare local species of medicinal plants

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Abstract. The article analyzes the results of growing new planting materials by tissue culture of some medicinal plants (Eminium regelii Vved., Angelica ternate Regel et Schmalh., Aconitum rotundifolium Kar. et Kir., Gentiana algida Pall., Rodiola heterodonta (Hook. f. et Thomson) Boriss.), found on the territory of the natural park Kara-Shoro of Kyrgyzstan. The conditions for growing callus cultures by the in vitro method were studied and the temperature optimum for growing cell cultures in the laboratory was identified. Callus cultures were grown on four types of nutrient media, which differ from each other in their component composition, and the preparation technique is the same.In the course of the study, it was found that the most universal nutrient medium is the Murashige-Skoog medium, in which callus formation is relatively more observed. The nutrient medium of Nitsch and Nitsch showed the lowest indicators of crop survival. Gamborg's and Eveleg's media showed good results, but root formation proceeded comparatively faster in White's nutrient medium. Planting schemes for medicinal plants and conditions for growing and development close to the ecological optimum have been developed. Based on the studies carried out, methods were proposed for creating a research and production complex for growing cell cultures of the State Natural Park (SNP) Kara-Shoro.

1 Introduction

There is a growing interest in herbal medicines all over the world, because their side effects, compared with synthetic ones, are minimal [1]. Recently, due to the unplanned collection and use of medicinal plant raw materials, there has been not only a rapid reduction in the ranges of such species, but the complete disappearance of many of them.

According to the monitoring carried out by the staff of the Kara-Shoro State Natural Park (SNP), located in the Uzgen district, some species of medicinal plants are on the verge of extinction (Table).

Table 1. Medicinal plants in the Kara-Shoro SNP, located on the territory of the Uzgen district

No.	Family	Genus	Species		
			Latin name	Russian name	Kyrgyz name
1	Araceae	*Eminium* L.	*Eminium regelii* Vved.	Regel's Eminium	Regel kuchalasy
2	Umbelliferae	*Angelica L.*	*Angelica ternate* Regel et Schmalh.	Angelica ternate	Archarot
3	Ranunculaceae	*Aconitum* L.	*Aconitum rotundifolium* Kar. et Kig.	Aconitum rotundifolium	Uu korgoshun (ak kodol)
4	Gentianaceae	*Gentiana* L.	*Gentiana algida* Pall.	Gentiana algida	Erbasyn
5	Crassulaceae	*Rhodiola* L.	*Rodiola heterodonta* (Hook. f. et Thomson) Boriss.	Rhodiola heterodonta	Altyn tamyr

One of the modern ways to solve the problem is to reduce the influence of people on the condition of widely used plants, and tissue culture is a way to obtain raw materials of such plants [2].

1.1 Plants used for callus cultures

Family Araceae. Genus *Eminium L. E. regelii* - Regel's Eminium - Kuchala. It is a rare endemic species of the Western Tien-Shan. It is a perennial plant, grows in forests, less often on shallow and clay soils in the mountains of the foothills and the lower part of the mountain belt. It is found in the Western Tien-Shan and the lower part of the Ferghana, Chatkal and At-Oinok ranges, the Northern Pamir-Alay. Reserves in nature are very limited. The species is on the verge of extinction. Included in the Red Book of the Kyrgyz USSR in 1985. The plant is widely used in folk medicine. From the literature data [3; 4; 5; 6; 7] it is known that infusions of tubers on koumiss or milk of the plant of the genus Eminium are used in Kazakh folk medicine in the treatment of patients with tuberculosis and oncological diseases. It was also found that the Regel's Eminium extract activates the antioxidant system in the liver and spleen, which indicates the possibility of restoring the protective systems of organisms exposed to radiation with a sublethal dose of 6 gy against the background of emotional stress due to natural antioxidants [8]. There is practically no information in the literature about the chemical composition of the Regel's Eminium plant [9].

Family Umbelliferae. Genus *Angelica* L. *A. ternate* Regel et Schmalh. – Angelica ternate – Archarot. It is a perennial plant, grows on scree, near glaciers, along the banks of streams in the Alpine belt. It is found in the Prifergan mountain region of Kyrgyzstan, the Inner Tien-Shan, Alay. The plant is widely used in folk medicine. Occasionally the plant is used in scientific phytotherapy [10].

Fig. 1. *Eminium regelii* - Regel's Eminium - **Kuchala**

Fig. 2. *A. ternate* Regel et Schmalh. – Angelica ternate – **Archarot**

The leaves are readily eaten by large and small cattle. The fruits are harmful and can cause poisoning. In the south of Kyrgyzstan, the plant is used in folk medicine as an anti-inflammatory [11; 12], antispasmodic, tonic [13], diuretic, diaphoretic. The use of medicinal plant preparations improves digestion [14; 15], normalizes the cardiovascular system, and has a calming effect on the nervous system of the body [10; 16].

Family Gentianaceae. *Gentiana* L., *G. algida* Pall. – Erbasyn. It grows on the kobresia wastelands, meadows of the Alpine belt.

Fig. 3. *G. algida* Pall. – **Erbasyn**

The species is distributed in Northern Kyrgyzstan - Issyk-Kul basin, Central and Western Tien-Shan, Prifergan mountain region of southern Kyrgyzstan, Inner Tien-Shan, Alay.

Antibacterial [20], anti-inflammatory [21], hemostatic [22] properties have been determined. Japanese researchers have isolated substances from gentian grass extracts capable of inhibiting the enzyme monoamine oxidase [23]. This opens up great prospects for the use of herbs in the treatment of diseases of the nervous system. In Tibetan medicine, the herbal concoction and infusion are recommended to be used as bitters, which will help improve the function of the stomach [24; 25]. Infusion of herbs and flowers of Gentiana algida is recommended for use in liver disease, gallbladder, gout, arthritis,

eczema, acute bronchitis, pulmonary tuberculosis, sore throat, aphonia, laryngitis, croup pneumonia [26; 27], as well as an antipyretic and detoxifying agent for various infectious and inflammatory diseases [21; 28].

Family Crassulaceae. Genus *Rhodiola* L., *R. heterodonta* (Hook. f. et Thomson) Boriss. (syn. *R. viridula* Boriss.) – Rhodiola heterodonta – Altyn tamyr.

Perennial plant. It grows on rocky slopes, in rock cracks, on placers in the Alpine belt, up to 4000 m above sea level. It is widespread in the Issyk-Kul basin, Central and Western Tien-Shan.

Figure 4. Species of the genus *Rhodiola* L. – Rhodiola rosea, Rhodiola heterodonta – Altyn tamyr – in the Prifergan district of Kyrgyzstan, the Inner Tien-Shan. It is a valuable medicinal plant, listed in the Red Book. An ecologically very vulnerable species, its range is rapidly shrinking.

Fig. 4. Species of the genus Rhodiola L. – Rhodiola rosea, Rhodiola heterodonta – Altyn tamyr

Currently, the issues of the raw material base for this plant are very relevant. Recently, there has been a discrepancy between the increasing needs of its procurement. Since ancient times, the rhizomes of this plant have been used in folk medicine as a tonic, in the form of an infusion and tincture as a means of relieving fatigue, increasing efficiency, as well as for anemia, impotence, scrofula, diseases of the stomach and nervous system [29; 30]. The possibility of antioxidant action is also considered [31; 32; 33], improvement of the state of the central nervous system [29; 30], the therapeutic effect of rhodiola extract has on the phenomena of parkinsonism, asthenia, etc. [31; 34; 35; 36].

In order to preserve and maintain biological diversity in the Kara-Shoro SNP, there is a need to develop new methods for obtaining raw materials of medicinal plants acceptable for use as raw materials in pharmaceuticals. In this aspect, in our opinion, it is advisable to use the well-known method of Andreev L.N., Gorbunov Yu.N. (2000) both insitu and exsitu [2]. The efficiency of exsitu plant conservation can be significantly increased by creating research and production complexes for growing cell cultures of medicinal plants by in vitro method.

Based on this, the purpose of this work was to develop acceptable methods for growing cell cultures of endangered species in order to preserve the biodiversity of medicinal plants of the Kara-Shoro SNP.

2 Materials and Methods

2.2 Scheme of the Experiment

The object of the study was the callus tissues of such medicinal, rare and endangered plant species of the Kara-Shoro SNP as *Eminium regelii*, *Rhodiola heterodonta*, *Gentiana algida*, *Angelica ternate*. To conduct the experiment, 20 minutes before the start of work, the internal volume of the laminar-box was irradiated with ultraviolet lamps. Sterile instruments were used only for one-time manipulation. Before repeated use, they were sterilized with alcohol again and burned. The nutrient media was sterilized by autoclaving at 1atm and a temperature of 121 °C.

The process of obtaining sterile plant material (free of epiphytic and rhizospheric microorganisms) consists of several stages. The first stage is pre-sterilization. Fragments of the stem, root and leaf were washed with running tap water and placed in alcohol (70% solution for 1 min). The second stage is sterilization. The pre-sterilized tissues were immersed in a sterilizing solution. All procedures related to the use of sterilizing substances were carried out under aseptic conditions (laminar-box). At the same time, not only the degree of purity of the plant material was taken into account, but also the preservation of its viability. One of the most harmless and effective agents is considered to be 5% calcium hypochlorite, which we used. The third stage is the washing of the object from the sterilizing solution (post sterilization). At the same time, the plant material was washed with 3-4 portions of sterile distilled water, keeping it in each portion for 10-15 minutes. The frequently used Murashige and Skoog medium was used for the experiment and for comparison – White's; Gamborg and Eveleigh; Nitsch and Nitsch medium [29, 31].

It has been shown that in vitro growth and development of plant tissues are greatly influenced by physical factors – light, temperature [30]. Sunlight and fluorescent lamps were used as a light source, with 16 hours of illumination per day, a temperature of 26 °C in the thermostat.

To obtain a callus culture (Figure 5), the prepared nutrient medium of 10 cm^3 was poured into Petri dishes and left in a laminar-box under ultraviolet light for 20 minutes. Passaging of plant cultures was carried out in a sterile box. Plant fragments 2-3 cm in size were extracted with aseptic tweezers and scalpel and placed in Petri dishes. As a rule, 1 explant from 4 plant species was placed in one Petri dish, 3 specimens from each species. Explants were cultured in the light at a temperature of 20-24 °C and grown in a thermostatically controlled room and a thermostat at a temperature of 26.6 °C. The resulting primary callus was weighed on analytical scales. The primary callus was separated from the necrotic parts, transferred to a fresh nutrient medium and further cultivated under the same conditions in a 16-hour photo period. The cycle of growing cultures was 36-40 days.

To compare experimental data on the choice of the optimal growing medium, nutrient media were prepared: White's, Gamborg and Eveleigh, Nitsch and Nitsch according to the same scheme as the Murashige and Skoog medium [31].

Fig. 5. Stages of obtaining a callus culture

3 Study Results and their Discussion

Callus cultures were grown on the four types of nutrient media mentioned above. As already noted, nutrient media differ from each other in component composition, the preparation technique is the same.

Ten explants from each plant species were grown on each nutrient medium: *Angelica* ternate*, Eminium* Regelii, *Gentiana algida, R. heterodonta*, obtained from the stem of plants. Callus cultures were grown in a thermostatically controlled room at 26 ± °C. Considering that the addition of hormones is of great importance for plant growth, growth-regulating hormones were added to the nutrient media. They play an indispensable role in the callus formation and cultivation of plant cells in the in vitro system. Auxins are necessary for the induction of callus formation, cell division and stretching of callus cultures, stimulation of root formation. Cytokinins are added to the nutrient medium to stimulate cell division in callus cultures, induction of stem organogenesis processes [29]. According to the method [31], we experimentally used 2,4 – dichlorophenoxyacetic acid (2,4-D) 0.00015 g, 6-benzylaminopurine (BAP) 0.000015 g, 1 – naphthyl acetic acid (NAC) 0.0003 g as hormones. For experiments during the first planting of callus cultures, the Murashige and Skoog medium was not autoclaved, and, as experimental data show, callus formation did not occur. This is due to the fact that some types of bacteria developed in the nutrient medium, which suppressed the growth of callus. The growth of callus cultures on the Murashige and Skoog medium with the addition of hormones on the example of *G. algida* Pall is shown in Table 2 and in Figure 1.

As can be seen from Table 2, callus formation did not occur on a nutrient medium that did not include hormones. In the work of the authors [32] in a nutrient medium, with the addition of hormones, callus formation was observed on the 3rd-5th day.

Table 2. Indicators of callus formation of *G. algida* Pall. – Gentiana algida

Name of plants	Duration of cultivation in days	With the addition of hormones	Without the addition of hormones
		Raw weight, mg	Raw weight, mg
G. algida Pall. – Gentiana algida	5	20	0.5
	10	30	0
	15	40	0
	20	50	0
	25	55	0
	30	75	0
	35	80	0
	40	82	0

In the present work, we observed that in 5 days the raw weight of *G. algida* was 20 mg. On 30-35 days, the growth of callus cultures begins to slow down, as the medium is depleted and there is a need to replant callus cultures to a new nutrient medium.

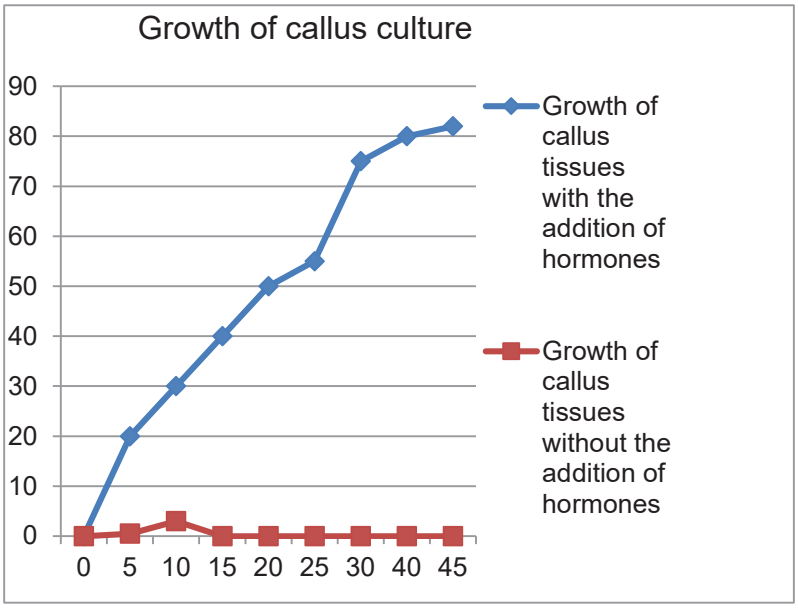

Fig. 6. Growth of callus culture of Gentiana algida, with and without the addition of hormones

The pH value for the nutrient medium varied from 5.6-5.8. The survival rate of callus cultures of plants varied and, as shown in Table 3, was different for each species. The survival and growth of callus is affected not only by the type of plant, but also by the nutrient medium.

As can be seen from Table 3 and Figure 2, the survival values of callus cultures have different indicators on nutrient media. On the Murashige and Skoog medium, the survival rates of callus cultures range from 70-90%. On the Gamborg and Eveleigh medium - from 60% to 80%, on the White's medium - from 50% to 70%. The lowest rates are observed in the Nitsch and Nitsch medium.

Table 3. Survival rates of callus cultures

Name of plants	Survival of callus tissues in %				
	Number of explants	Murashige and Skoog medium	Gamborg and Eveleigh medium	White's medium	Nitsch and Nitsch medium
A. *ternate* – Angelica ternate	10	70%	80%	60%	30%
Eminium Regelii – Regel's Eminium	10	80%	70%	50%	30%
G. algida – Gentiana algida	10	90%	80%	70%	20%
R. heterodonta – Rhodiola heterodonta	10	80%	60%	70%	40%

Based on this, we have concluded that the most universal nutrient medium is the Murashige and Skoog medium, on which callus formation occurs relatively more. The Gamborg and Eveleigh medium gives good results. On White's nutrient medium, root formation occurred relatively faster. The nutrient medium with the lowest survival rates is the Nitsch and Nitsch nutrient medium. Callus cultures were characterized by the following morphological features: slow callus growth (the growth cycle averages 60-80 days). The callus is light-colored in the first weeks, then darkened. No signs of morphogenesis were revealed during visual analysis with naked eye.

Callus tissues can be grown as much as you like, if you replant them every 24-30 days to a fresh nutrient medium, because 3-4 weeks after the depletion of the nutrient medium, callus cells necrotize, in consequence of which, the callus changes color to a dark shade, losing its medicinal properties.

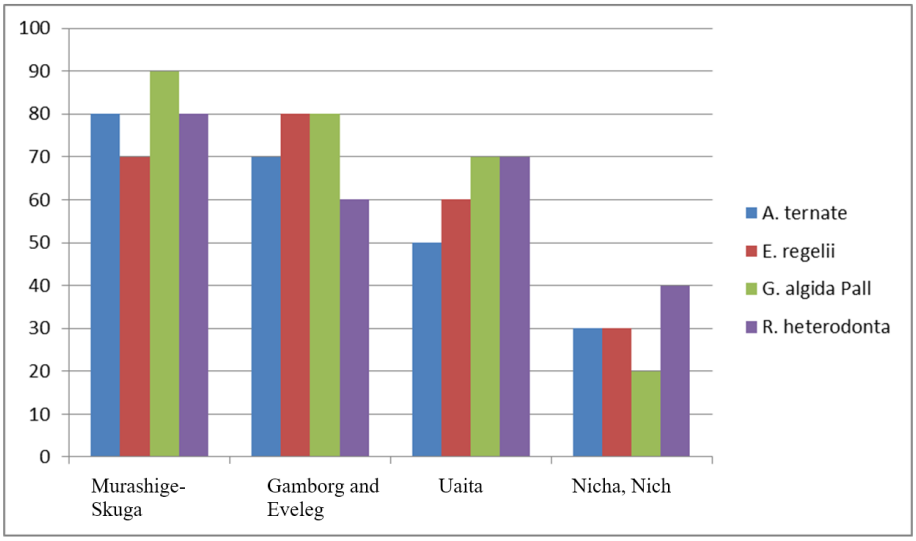

Fig. 7. Survival diagram of callus cultures of plants on various nutrient media.

3.1 Discussion

It is noted that the reduction in the reserves of the most common types of medicinal plants of the Kara-Shoro State Natural Park is caused by the impact of such anthropogenic factors

as unplanned collection of raw materials, improper agricultural development of territories, as well as overgrazing, etc. In our opinion, the main way to preserve medicinal plants of the Kara-Shoro State Natural Park is to create cultural sites in places where conditions are close to their natural habitats. The planting material in such cases is cultural materials obtained in tissue culture. This way, firstly, allows to preserve natural thickets and, secondly, it can partially or completely satisfy the need for one or another type of plant raw materials. Due to the growing interest of the population in folk treatment and gardening, the issue of growing medicinal plants on household plots becomes urgent. To meet their needs, it is necessary to train amateur gardeners to grow seedlings on their plots obtained using tissue culture using the methods we have proposed.

Thus, with the help of cell engineering methods, it is possible to restore and preserve the natural reserves of endangered medicinal plants in the Kara-Shoro Park. In the future, by expanding the plantations outside the Kara-Shoro Park, it is possible to establish production for the preparation of medicinal plants obtained by the use of tissue culture.

4 Conclusions

1. The conditions of growing callus cultures by in vitro method have been studied. The temperature optimum for growing cell cultures in laboratory conditions has been determined.

2. In order to reduce the pressure on natural reserves, rational use of natural resources and preservation of medicinal plants, their cultivation by the method of cell cultures is proposed. Schemes for growing medicinal plant planting materials have been developed and conditions for growth and development have been created that are close to the ecological optimum.

3. Recommend the method of cellular engineering for obtaining raw materials for the production of medicines, essential oils, etc. The use of tissue culture will to some extent alleviate the problems of conservation of the reserves in nature of some species of endangered plants in the territory of the Kara-Shoro National Park.

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