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OBTAINING ASEPTIC CULTURE *IN VITRO* OF *RHODIOLA SEMENOWII L.* FROM VARIOUS TYPES OF EXPLANTS

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ABSTRACT

In vitro cultivation of callus cells allows for the stable production of secondary metabolites, being an alternative to the use of medicinal plant biomass from natural populations. Standardization of cultivation protocols and technological processes for the synthesis of secondary metabolites *in vitro* conditions determines significant interest in the use of tissue culture methods (PTC – *Plant Tissue Culture*). The aim of the study was to obtain an aseptic culture of *Rhodiola semenowii in vitro* from various types of explants, as well as the effect of the hormonal composition of the nutrient medium on the induction of callusogenesis in the culture of isolated organs of *R. semenowii*. As a result of the research, the optimal mode of obtaining *R. semenovii* aseptic cultures from various explants was determined. It has been shown that the use of 70% EtOH in combination with 0.01% $HgCl_2$ or 20% NaClO makes it possible to obtain aseptic cultures from any type of *R. semenovii* explants. It has been established that the type of explant is *R. semenovii* influenced both the frequency of callus formation and the beginning of its formation. The least optimal types of *R. semenovii* explants are stem and rhizome segments, the frequency of callusogenesis varied from 5.8% to 75.6%. The induction of callusogenesis also depended on the hormonal composition of the nutrient medium: the highest percentage of callus formation in all types of explants was on media containing 0.5mg L^{-1} thidiazuron (TDZ) + 1.0 mg L^{-1} 1-naphthaleneacetic acid (NAA) and 0.5mg L^{-1}2,4D + 2.0 mg L^{-1} 6-Benzylaminopurine (BAP).

Key words: *Rhodiola semenowii* L., medicinal plant, explant, sterilization, phytohormones, *in vitro* culture.

INTRODUCTION

Both the antioxidant and anti-inflammatory activity *in vivo* and *in vitro*, as well as the regenerative and antitumor properties of extracts of various species of the genus *Rhodiola*, are beyond doubt, which is due to the presence of various biologically active components in their composition. The immunoregulatory and antidepressant properties of the extracts allow the use of plants of the genus *Rhodiola* in folk and traditional medicine to increase the adaptive potential of the human [1, 2]. The most studied species are such as *R. rosea* and *R. crenulata*, the research results are protected by numerous patents [3]. The most valuable phytonutrient characteristic of all species of *Rhodiola* sp. is phenylethanoid salidroside, which has a wide range of biological properties for medicine, cosmeceuticals and the food industry [4]. However, the high popularity of *R. rosea* predicts an increase in demand for its phytopreparations, and this has a negative impact on the availability of raw materials due to a reduction in natural reserves. The need to preserve the biodiversity of *R. rosea* determines the need for state protection and control over its harvesting in Kazakhstan [5]. In order to ensure the availability of plant raw materials for the needs of medicine, research on the search for other alternative sources of BAS, similar in biological value to *R. rosea*, has recently become relevant.

There are 11 species of *Rhodiola sp.* growing in Kazakhstan, of which the poorly studied species is *Rhodiola semenowii* (Regel & Herder) *Borissova* [6]. The plants of *Rhodiola semenowii* are vegetatively reproducing short-rooted succulent perennials growing in the alpine belt of Zailiysky and Kungei Alatau at an altitude of up to 3500 m above sea level [7]. They prefer high soil moisture and a sunny location [8, 9]. It is a perennial wild herbaceous plant in the family *Crassulaceae*, growing along the banks of mountain rivers, up to 60 cm high with thin linear leaves and a thick branched rhizome. Reproduction occurs both by seeds (under favorable conditions) and vegetatively by dividing rhizomes. In one location *R. semenovii* plants are able to live for several years. A distinctive feature of this species is the presence of hermaphrodite flowers, while for most other species *Rhodiola* sp. The presence of dioecious same-sex flowers is characteristic [10, 11].

R. semenovii plants contain proanthocyanidins, coumarins, flavonic glycosides and organic acids, tannins of the pyrogallic and pyrocatechin groups [12]. The studies have shown that the use of *R. semenovii* extracts significantly increases endurance due to its antihypoxic properties [13, 14, 15]. It was also found that the roots of *R. semenovii* contain cyanogenic glucosides - rhodiocyanoside A and lotaustraline which have lipid-lowering, hypocholesterolemic and anti-inflammatory properties [16, 17]. A pronounced therapeutic effect of rhodioflavonoside contained in root extract of *R. semenowii* has been revealed, which makes it possible to use it to normalize the pituitary gland and correct pathological states associated with a decrease in thyroid function [18].

The advantages of using PTC to obtain secondary metabolism products *in vitro* are the possibility of using GLP and GMP standards, the absence of dependence on external conditions, and the sterility of raw materials. But the main strength is the preservation of the number and biodiversity of plant populations in the wild nature [19, 20]. The development of methods for callus and suspension cultivation of Rhodiola species *in vitro* makes it possible to eliminate the shortage of medicinal raw materials in natural populations, as well as to prevent damage to rhizomes of *Rhodiola* sp. plants by rodents and pathogenic organisms during plantation cultivation [21]. Modern *in vitro* cultivation technologies eliminate the influence of environmental and seasonal factors, as well as significantly reduce threats to natural biodiversity. With regard to the production of BAS in *in vitro* systems in valuable species of *Rhodiola* sp. there are many successful examples both with the use of callus, suspension cultures, and in the «hairy

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root» culture [22, 23].

Studies show that dedifferentiated tissues of *Rhodiola* sp. are capable of producing salidroside *in vitro*, depending on the type of explant, methods and duration of its sterilization, and cultivation conditions. This is due to the fact that morphological differentiation of plant cells and biochemical processes *in vitro* are often interrelated.

Use of Plant growth regulators (PGR) for callus tissue proliferation in combination with various elicitors can determine cell differentiation and direction of secondary metabolism in various in vitro systems [24]. It should be noted that the induction of callus formation is the main stage of indirect organogenesis. This stage is necessary for obtaining suspension cultures of plants for industrial production of bioactive substances. In this regard, the creation of a stable and highly dynamic callus system is important for the subsequent synthesis of secondary metabolites *in vitro*.

The aim of the research was to optimize the methods of sterilization of various explants of *Rhodiola semenowii* to obtain an actively proliferating callus line.

MATERIALS AND METHODS

The seeds and plants of *Rhodiola semenowii* collected in the places of their natural growth in the Great Almaty Gorge (Almaty region) at an altitude of 2500 m above sea level (N 43°02.503'; E 76°59.744') (Figure 1).

Sterile seedlings, as well as segments of stems, leaves and rhizomes of adult plants, were used as explants for the induction of callusogenesis.

To obtain aseptic cultures of *R. semenovii*, 8 different sterilization protocols were used using various sterilizing agents: ethanol (EtOH), mercuric chloride ($HgCl_2$), potassium permanganate ($KMnO_4$), sodium hypochlorite (NaClO) and hydrogen peroxide (H_2O_2) and exposure time (Table 1).

Previously, all explants were thoroughly cleaned, then rinsed under running water for 4 hours. After each disinfectant, a 3-fold rinsing with sterile water was carried out for 15 minutes to remove antiseptic residues. Next, the sterilized explants (seeds) were placed on the surface of a solid nutrient medium ½ MS with the addition of 30 g L^{-1}sucrose and 10 mg L^{-1}gibberellic acid. Segments (cotyledon leaves and hypocotyl) of 2-week-old seedlings were further used as explants for

Figure 1. Plants of *R.semenovii* in the Big Almaty gorge (Almaty region)

Table 1. Sterilization schemes of *R. semenovii* explants

№	Sterilizing agent (%) and exposure time (min)				
	EtOH	$HgCl_2$	H_2O_2	NaClO	$KMnO_4$
1	70% 3'	-	3% 20'	-	-
2	70% 3'		3% 30'	-	-
3	70% 3'	0,01% 20'	-	-	-
4	70% 3'	0,01% 30'	-	-	-
5	70% 3'	-	-	20% 20'	-
6	70% 3'	-	-	20% 30'	-
7	70% 3'	-	-	-	0,1% 20'
8	70% 3'	-	-	-	0,1% 30'

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Table 2. Concentration of various phytohormones for induction of *R. semenovii* callusogenesis

Environment	Concentration of exogenous phytohormones, mg L^{-1}				
	TDZ	NAA	2,4-D	Kin	BAP
MS	-	-	-	-	-
MS1	0,5	1,0	-	-	-
MS2	-	2,0	1,0	-	-
MS3	-	-	0,5	-	2,0
MS4	-	--		0,2	2,0

the induction of callusogenesis [25].

The effectiveness of sterilization schemes was determined by the number of aseptic viable explants on the 7th day of cultivation (necrotized, infected explants were not taken into account).

Tissue explants were placed on the surface of a solid MS nutrient medium containing 30 g L^{-1}sucrose, with the addition of various exogenous growth regulators. Experiments on the selection of media for callus formation and further cultivation of callus tissues were carried out on Murashige and Skuga media with the addition of various phytohormones (Table 2).

The explant cups were placed in a climate chamber at a temperature of 26 ° C, light mode 16/8. During the studies, the frequency of callusogenesis (%), the onset of callus formation, as well as the color and structure of primary callus were evaluated [26].

RESULTS

Optimization of the sterilization protocol for Rhodiola semenowii explants

Obtaining sterile and viable primary explants has always been a major challenge for *in vitro* culture, especially for plants taken from the wild. Elimination of epiphytic microflora is not a major problem, but it is more difficult to get rid of latent infection in a latent form, which can lead to necrosis and death of viable explants in the future. One solution may be the sequential use of several sterilizing agents, each with a specific effect on pathogenic microflora [21].

As a result of the study of 8 sterilization protocols, differing in sterilizing agents, their combination and exposure time, it was found that there was no specific explant type reaction depending on the sterilization scheme (Table 3).

As a result of our research, it was found that the maximum percentage of aseptic explants (seeds, green plants, rhizome buds) was achieved using 3, 4 and 5 sterilization schemes, including treatment with 70% ethanol for 3 minutes, 0.01% mercury chloride (20-30 minutes) and 20% sodium hypochlorite (20 minutes). When using these sterilization schemes, the percentage of aseptic viable seeds varied from 82.4 to 92.5%, green plants from 60.0 to 81.1%, rhizome segments from 73.1 to 85.5%. The use of 0.1% potassium permanganate (scheme No. 7, No. 8) was not effective enough, since it was not possible to achieve a sterilizing effect on the microflora, almost all explants in these variants were contaminated with pathogenic microorganisms. With an increase in the exposure time of NaClO to 30 minutes (scheme No. 6), the number of darkened necrotic explants increased and their viability values significantly decreased.

Thus, we have selected sterilization schemes (schemes No. 3, 4 and 5), which can be effectively used to obtain sterile *R. semenovii* explants of various organ accessories.

Introduction of plant tissues and organs into culture in vitro

When plant tissues and organs are introduced into culture *in vitro*, as a result of dedifferentiation of the cells of the initial explant and their further division, callus tissue is formed. The ability to callus formation is largely determined by the type and concentration of auxins and cytokinins. The selection of phytoregulators used is one of the most important factors influencing the processes of callusogenesis, as well as the production of secondary metabolites [20].

To induce callus formation, we used various types of explants - segments of sterile seedlings (cotyledonous leaves and hypocotyl), segments of stems and leaves of green plants, as well as fragments of rhizomes (Figure 2).

Studies have shown that there were no signs of callus for-

Table 3. Choosing the optimal sterilization scheme for *R. semenovii* explants

Sterilization scheme	**Aseptic viable explants, %**		
	Seeds	**Mature plants**	**Rhizomatous segments**
1	47,5	43,8	38,5
2	49,4	48,6	40,5
3	92,5	81,1	85,5
4	82,4	61,8	73,1
5	83,1	60,0	83,6
6	34,0	39,6	36,4
7	7,0	7,1	8,7
8	3,4	4,3	6,3

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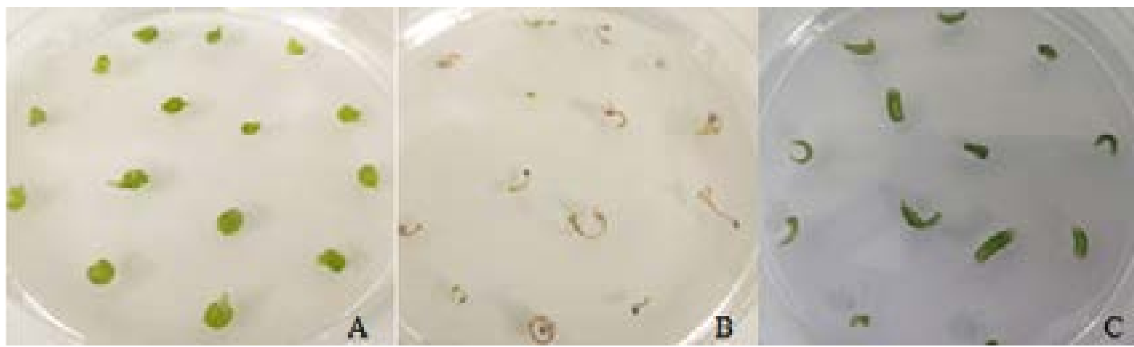


A - cotyledonous leaves; B – hypocotyl; C - leaves

Figure 2. Different types of explants for induction of *R. semenovii* callusogenesis

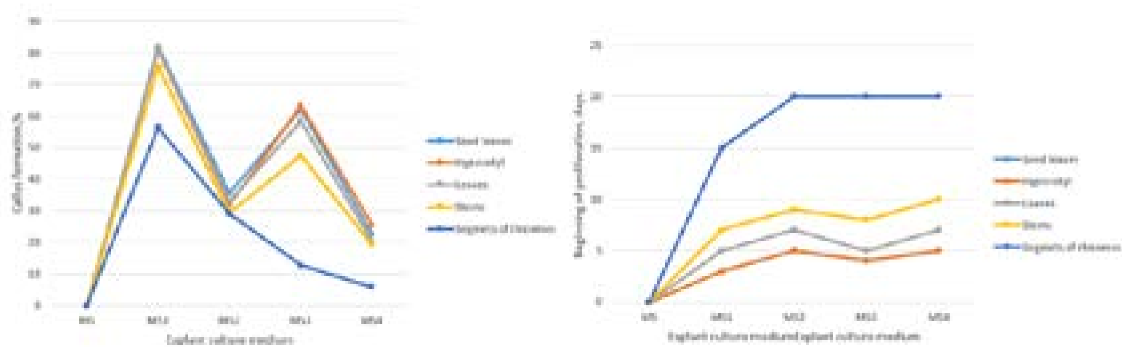
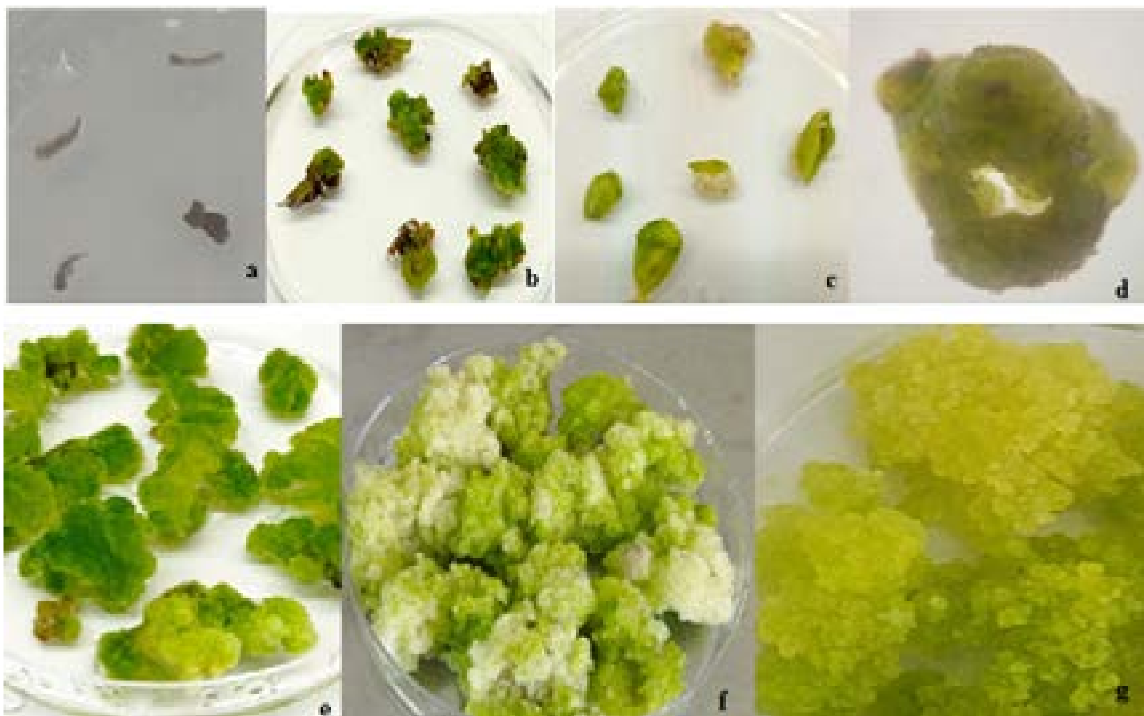


Figure 3. The effect of various types of explants and phytogorones on the induction of *R. semenovii* callusogenesis



a – necrosis of stem and leaf explants; b - d – initiation of callus formation from hypocotyl segments (b), leaves (c), stems (d); e – active accumulation of biomass of leaf explants after 30 days; f – active accumulation of biomass of leaf explants after 45 days; g – formation of loose callus of *R. semenovii* for subsequent suspension cultivation

Figure 4. Induction of callusogenesis in various types of *R. semenovii* explants

mation on the hormone-free MS medium: no hydration of explants, their proliferation and growth were noted. After 4 weeks of cultivation, the tissue explants had a dark brown color, and after 8 weeks we noted necrosis.

The period of callus formation from the introduction of primary explants to the proliferation of callus cells depended on tissue and organ affiliation. Segments of cotyledon leaves, hypocotyles of sterile seedlings and leaves of adult plants reacted the fastest to the effect of phytohormones (Figure 3).

Thus, the beginning of active cell proliferation in cotyledonous leaves and hypocotyl began on 3-5 days, in leaves on 5-7 days, on stem segments, callus formation began on 7-10 days. On the rhizomes, later periods of the onset of callus formation are noted - 15-20 days.

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Depending on the variant of the medium and the type of explant, the callus-forming ability varied from 5.8% to 81.8%. The most active processes of cell dedifferentiation occurred on a medium containing 0.5mg L^{-1}TDZ and 1.0 mg L^{-1}NAA (medium MS1) in all types of explants, the intensity of callus formation ranged from 56.3% to 81.8%. The combined use of auxin 2,4-D and cytokinin BAP (MS3 medium) also contributed to an increase in the frequency of callus formation for all types of explants and ranged from 12.6% to 63.3%. It was noted that when using the rhizome as an explant, the optimal combination of phytohormones in the medium was 0.5mg L^{-1}TDZ and 1.0 mg L^{-1}NAA (medium MS1) and 2.0 mg L^{-1}NAA and 1.0 mg L^{-1}2,4-D (medium MS2). In general, it can be said that rhizome segments are the most difficult type of explants, since in addition to the weak ability to callus formation, there is also the production of polyphenols into the nutrient medium. This makes it difficult to use them as explants and requires additional research.

The formed calluses were bright green in color, dense compact with a granular structure and the presence of green meristematic zones (Figure 4).

Subsequently, the calluses were passioned for the accumulation of biomass and subsequent stages of research. Actively proliferating callus lines of *Rhodiola* sp. used to study the peculiarities of the biosynthesis of some secondary metabolites.

DISCUSSION

Obtaining an actively proliferating callus line is a difficult task, since the efficiency of the process is influenced by many factors – and above all, the type of explant, the sensitivity of which to disinfectants affects its viability and the production of secondary metabolites *in vitro*.

Studies of isolated tissue and organ cultures of various *Rhodiola* species mainly concern issues of clonal micropropagation, as well as callus induction and morphogenesis in order to preserve the biological diversity of this valuable medicinal plant. Based on studies conducted by phytochemists and pharmacologists, it was found that plants of the genus *Rhodiola*, including the species *R. semenovii*, contain proanthocyanidins, coumarins, flavonic glycosides, organic acids, as well as tannins of the pyrogallic and pyrocatechin groups. Alkaloids, glycosides, flavonoids, ascorbic, malic and succinic acids, sugars, wax, rutin and tannins were also found in these plants [15].

However, the high popularity of various types of *Rhodiola* predicts an increase in demand for its phytopreparations, and this has a negative impact on the availability of raw materials due to a decrease in natural reserves, since the resumption of natural populations takes at least 10-15 years [21]. The technology *in vitro* cultivation of plant organs and tissues has recently become particularly relevant due to the impact of changing climate conditions on wild populations of medicinal plants subject to uncontrolled collection of raw materials. Plant tissue culture has the advantage of replacing scarce raw materials of valuable medicinal plants, or plants that are difficult to grow under *ex situ* conditions. It also makes it possible to obtain a sufficiently large amount of phytomass free from pathogenic microflora, herbicides, pesticides, and heavy metals. Callus cultures provide an opportunity to synthesize new compounds that are not produced by the corresponding plants in nature, and also allow you to control the biosynthesis of target products by modifying the cultivation conditions and the composition of the nutrient medium. In addition, they open up prospects for industrialization and reducing the cost of producing certain biologically active substances, the synthesis of which is either not developed at the moment or is very expensive [20, 23]. The cultivation of plant organs and tissues holds great potential for the industrial use of bioactive metabolites for the pharmaceutical, cosmetic and food industries, and PTC are also capable of producing new substances with unknown properties that can be used for therapeutic purposes [21]. The potential use of callus is not limited to the production of secondary metabolites; PTC can potentially be used to regenerate organs and entire plants with desired properties through the use of new genome editing technologies.

Callus cultures can also be converted into single-celled suspension cultures to produce the desired secondary metabolites. This process takes place under controlled conditions, which eliminates the influence of various environmental factors, seasonal fluctuations, infection with various diseases and pests, and geographical restrictions. All this makes it possible to produce secondary metabolites of consistently high quality.

CONCLUSION

Thus, as a result of the research, the optimal mode of obtaining aseptic cultures from various types of *R. semenovii* explants was determined, including sequential treatment with 70% EtOH for 3 minutes in combination with 0.01% $HgCl_2$ (20-30 minutes) or 20% NaClO (20 minutes). When using these sterilization schemes, the percentage of aseptic viable seeds ranged from 82.4% to 92.5%, green plants from 60.0% to 81.1%, rhizome segments from 73.1% to 85.5%.

It was found that the type of callus formation frequency and the time of the beginning of the formation of callus cells largely depends on the type of explant, the most effective callus formation occurs when using segments of sterile seedlings and leaves of adult plants. The induction of callusogenesis directly depended on the hormonal composition of the nutrient medium. In all tested media, callus cells were formed, the highest intensity of callus formation was higher on media MS1 and medium MS3, and amounted to 81.8% and 63.3%, respectively, this combination of phytohormones proved to be the most effective for all types of explants. Rhizome segments are the most complex type of explants, the frequency of callus formation of which was the lowest in the experiment among all types of explants used.

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ПОЛУЧЕНИЕ АСЕПТИЧЕСКОЙ КУЛЬТУРЫ РОДИОЛЫ СЕМЕНОВА *(RHODIOLA SEMENOWII L.)* В УСЛОВИЯХ *IN VITRO* ИЗ РАЗЛИЧНЫХ ТИПОВ ЭКСПЛАНТОВ

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АННОТАЦИЯ

Культивирование каллусных клеток *in vitro* позволяет обеспечить стабильную продукцию вторичных метаболитов, являясь альтернативой использованию биомассы лекарственных растений из природных популяций. Стандартизация протоколов культивирования и технологических процессов синтеза вторичных метаболитов в условиях *in vitro* обусловливает значительный интерес к использованию методов культуры тканей (PTC – Plant Tissue Culture). Целью исследования было получение асептической культуры *Rhodiola semenowii in vitro* из различных типов эксплантов, а также влияние гормонального состава питательной среды на индукцию каллусогенеза в культуре изолированных органов *R. semenowii*. В результате проведенных исследований определен оптимальный режим получения асептических культур *R. semenovii* из различных эксплантов. Показано, что использование 70% EtOH в сочетании с 0,01% $HgCl_2$ или 20% NaClO позволяет получать асептические культуры из любых типов эксплантов *R. semenovii*. Установлено, что тип экспланта *R. semenovii* оказывал влияние как на частоту образования каллуса, так и на начало его формирования. Наименее оптимальными типами эксплантов *R. semenovii являются сегменты стеблей и корневищ, частота каллусогенеза варьировала от 5,8% до 75,6%.* Индукция каллусогенеза также зависела от гормонального состава питательной среды: наибольший процент каллусообразования у всех типов эксплантов был на средах, содержащих 0,5 мг/л тидиазурона (ТДЗ) + 1,0 мг/л 1-нафтилуксусной кислоты (НУК) и 0,5 мг/л 12,4Д + 2,0 мг/л 6-бензиламинопурина (БАП).

Ключевые слова: *Rhodiola semenowii L.*, лекарственное растение, эксплант, стерилизация, фитогормоны, культура *in vitro*.

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ӘРТҮРЛІ ЭКСПЛАНТ ТҮРЛЕРІНЕН *IN VITRO* ЖАҒДАЙЫНДА СЕМЕНОВА ҚЫЗҒЫЛТ СЕМІЗОТТЫҢ *(RHODIOLA SEMENOWII L.)* АСЕПТИКАЛЫҚ КУЛЬТУРАСЫН АЛУ

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ТҮЙІН

Каллус жасушаларын *in vitro* жағдайында өсіру табиғи популяциялардан дәрілік өсімдіктердің биомассасын қолдануға балама бола отырып, екіншілік метаболиттерді тұрақты өндіруге мүмкіндік береді. Өсіру протоколдарын және екіншілік метаболиттерді синтездеудің технологиялық процестерін *in vitro* стандарттау ұлпа культурасы әдістерін (PTC - Plant Tissue Culture) қолдануда айтарлықтай қызығушылық тудырады. Зерттеудің мақсаты әртүрлі типтегі экспланттардан *Rhodiola semenowii in vitro* асептикалық культурасын алу, сонымен қатар қоректік ортаның гормональдық құрамының *R.semenowii* оқшауланған мүшелері культурасында каллузогенез индукциясына әсерін зерттеу болып табылады. Жүргізілген зерттеу нәтижесінде әртүрлі экспланттардан *R.semenovii* асептикалық культураларын алудың оңтайлы режимі анықталды. 70% EtOH 0,01% $HgCl_2$ немесе 20% NaClO біріктіріп қолдану *R.semenovii* экспланттарының кез келген түрінен асептикалық дақылдарды алуға мүмкіндік беретіні көрсетілген. *R.semenovii* эксплантының түрі каллус түзілу жиілігіне де, оның қалыптаса бастауына да әсер еткені анықталды. *R.semenovii* сабақтары мен тамырсабағының сегменттері оңтайлы емес эксплант түрлері болып табылады, каллузогенездің жиілігі 5,8%-дан 75,6%-ға дейін өзгерді. Каллусогенездің индукциясы қоректік ортаның гормондық құрамына да байланысты болды: экспланттардың барлық түрлерінде каллус түзілудің ең жоғары пайызы құрамында 0,5 мг/л тидиазурон (ТДЗ) + 1,0 мг/л 1-нафтилсірке қышқылы (NAA) және 0,5 мг/л 12,4Д + 2,0 мг/л 6-бензиламинопурин (БАП) бар орталарда болды.

Кілтті сөздер: *Rhodiola semenowii L.*, дәрілік өсімдік, эксплант, зарасыздандыру, фитогормондар, *in vitro* культурасы.