

Introduction of *hyssopus officinalis* L. into in vitro culture to optimize the conditions for obtaining callus tissues and microclonal propagation as a promising method of innovative agrobiotechnologies

Elena Maslova^{1*}, Natalya Gulya², Tatyana Perelugina¹, Valeria Semykina¹, and Elena Kalashnikova³

¹Belgorod State National Research University, Belgorod, Russia

²Biotech company «Biocad», Moscow, Russia

³Russian State Agrarian University – Moscow Timiryazev Agricultural Academy

Abstract. The sterilization process of plant explants of *H. officinalis* was optimized when introduced into an in vitro culture, the most effective sterilization modes, optimal sterilizing agents, their exposure time and concentration were selected. Callus tissues and mini-plants of *H. officinalis* were obtained in vitro and the most optimal nutrient media were determined both for microclonal propagation and for the induction of callus tissue *H. officinalis*, which can be further used for mass cultivation of cell and culture and obtaining safe bio-additives with active substances for livestock and crop production as a part of the development of modern agrobiotechnologies.

1 Introduction

Modern agrobiotechnology is currently the main priority area of development of the Russian Federation. To increase the efficiency of crop production and obtain greater productivity and yield of the target product, innovative agricultural and biotechnological methods are used. So, for example, microclonal propagation is used for further mass propagation and cultivation of high-quality healthy plants by the traditional method in field and greenhouse conditions. The same method of cell and tissue culture can be used to obtain callus tissue of plants and to cultivate their cellular plant biomass in laboratory conditions and bioreactors in industrial biotechnological enterprises to obtain the target product, containing valuable biologically active substances or components with antibacterial, antimicrobial, antitumor and immunomodulating properties. They can be used in animal husbandry as a biological supplement and in crop production as a biological product that reduces the use of chemical additives and drugs that increase the immunity of both plant and animal organisms. One of such new promising sources is the representative of the Lamiaceae family - *Hyssopus officinalis* L. is a representative of the genus *Hyssopus*.

H. officinalis is a perennial semi-shrub plant that grows under natural conditions in the southern regions of the Russian Federation, the Caucasus, the Black Sea region, the Crimea, Altai, Central Asia, Europe and North Africa [1-2].

H. officinalis is a honey plant, as well as a medicinal and a spicy aromatic plant. It is included in the pharmacopoeias of France, Sweden, Germany, Romania,

and Portugal [3]. It contains essential oil, sesquiterpenes, tannins, oleanolic and ursolic acids, resins, mineral salts, organic acids, flavonoids [2, 4].

It is known that in the flowering phase, the aerial mass of *H. officinalis* contains up to 1.5% of essential oil in terms of dry weight; flavonoids (apigenin, luteolin, quercetin), their glycosides and phenolic acids (chlorogenic, protocatechuic, ferulic, p-coumaric, rosmarin and gentisin); Vitamin C and carotene, along with many other substances [5-7]. The essential oil of *H. officinalis*, which is obtained in many countries of the world, is of great interest [8-9]. The chemical composition of *H. officinalis* essential oil was studied in various parts of the world, and according to the results of most studies, the main components are pinocamphone and isopinocamphone [5-7, 10-13]. The composition of the essential oil and the quantitative content of various compounds differ depending on soil-climatic and genetic factors [13]. Nevertheless, the main components characteristic of this plant are pinocamphone, isopinocamphone, β pinene, sabinene, myrcene, β -fellandren, linalool, myrtenol, elemol and germacren-D. Thymol and methyl acetate chemotypes of *H. officinalis* also grow in Iran [14-15]. *Hyssopus* vegetable raw materials and essential oils are used in perfumes and cosmetics (aromatic components of perfumes, cosmetics, soaps), food (spice and fragrant seasoning for flavouring cold snacks, meat and fish dishes, sauces, alcoholic beverages) and the pharmaceutical industry. *Hyssopus* has excellent phytoncidal properties [16-17].

*Corresponding author: maslova@bsu.edu.ru

Of particular value are the leaves of the plant. *Hyssopus officinalis* has anti-inflammatory, antiseptic, antitussive effects. It stimulates digestion and relieves spasms of the smooth muscles of the gastrointestinal tract [1, 18]. *Hyssopus* infusions are used medicinally for diseases of the upper respiratory tract, cough, bronchitis, bronchial asthma, pneumonia and pulmonary tuberculosis, angina pectoris, neurosis, rheumatism, polyarthria, for the treatment of bruises, wounds, skin diseases [1]. This species has an expectorant, antispasmodic, decongestant, tonic effect, and some of its species exhibit a strong antiviral effect, especially against the herpes virus [18].

In experiments, conducted by scientists, *Hyssopus officinalis* exhibits antibacterial, antimycotic and antiprotozoal activity [19], and also has fungicidal properties [20].

Overall, it is clear that this culture is increasingly attracting the attention of scientists, due to the accumulation of a large number of biologically active compounds in plant tissues, which has been used in the pharmacy and food industry, and can also be used in modern agrobiotechnologies. Therefore, the relevance of studying of *H. officinalis* in an in vitro culture is evident and it is one of the priority areas of modern agrobiotechnology. To date, data on the study of the influence of cultivation conditions on clonal micropropagation and on the callus and morphogenesis of *H. officinalis* are not numerous [21-24], and the optimization of the in vitro cultivation process and the detailed study of this plant for the purpose of agrobiotechnology remains an urgent task.

The aim of our study is to optimize the sterilization process and the conditions for obtaining callus tissues and microclonal propagation as a promising culture for use in modern agrobiotechnologies.

2 Experimental

For research, the plant material of intact *H. officinalis* plants growing on the territory of the Botanical Garden

of the National Research University “BelSU” was collected. To introduce into in vitro culture and to obtain seedlings and mini plants, plant seeds were used as plant explants.

Sterilization was carried out in a stepwise manner according to generally accepted methods [25], using various disinfectants.

As sterilizing solutions, we used: sodium hypochlorite in a concentration of 5-15% and 2.5-7.5%; lysoformin 3000 - 3%, 5% and 10%; biocide - 3%, 5% and 10%; silver nitrate - 0.1% and 0.5%; chloramine B at a concentration of 5% and 10%. Sterilizing solutions were left to expose to plant explants for 10, 20 and 30 minutes. At first, the plant material, washed in a soap solution, was placed in 70% ethyl alcohol for 1 minute, and then in a sterilizing solution of a certain concentration and for a certain time and after that it was washed three times in sterile distilled water. Sterile seeds were placed on the hormone-free Murashige-Skoog nutrient medium [26].

Further cultivation was carried out on a modified agar Murashige-Skoog medium [26] with a different composition of phytohormones (Table 1).

Nutrient media were made according to generally accepted in vitro culture methods for plant cells and tissues (the hormone-free Murashige-Skoog medium was used as a control) [27].

All work and manipulations with cultures were carried out under aseptic conditions in the Lamsystems laminar box of protection class II, type A2, according to generally accepted sterility practices when working with cell and tissue cultures [27-28].

After seed germination the seedlings were transferred, under aseptic conditions, to nutrient media containing various concentrations of phytohormones and cultivated in a light room at a temperature of 23 °C with observance of the day / night regime: 16/8 hours for 30 days.

Table 1. Composition of modified Murashige-Skoog nutrient media

Components of the medium	MS ₁	MS ₂	MS ₃	MS ₄	MS ₅	MS ₆	MS ₇	MS ₈
	Volume per 1 L							
Macrosalt stock solutions, ml	100	100	100	100	100	100	100	100
Microsolt stock solutions, ml	1	1	1	1	1	1	1	1
Fe-chelate, ml	5	5	5	5	5	5	5	5
CaCl ₂ , ml	5	5	5	5	5	5	5	5
Vitamins: PP, mg	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
B ₁	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
B ₆	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Mesoinositis, mg	100	100	100	100	100	100	100	100
Glycine, ml	2	2	2	2	2	2	2	2
α-Naphthaleneacetic acid (NAA), mg	-	-	-	-	-	0.5	2	-
Indole-3-acetic acid (IAA), mg	-	-	1	-	2	-	2	3
Indole-3-butyric acid (IBA), mg	-	-	-	1	-	-	-	-
Kinetin, mg	-	-	-	-	0.2	0.1	1	2
6-benzylaminopurine (6-BAP), mg	1	3	-	-	-	1	1	-
2,4-Dichlorophenoxyacetic acid (2,4-D), mg	-	-	-	-	-	-	2	-
Sucrose, g	20	20	20	20	20	20	20	20
Agar-agar, g	7	7	7	7	7	7	7	7

The quality of seedling growth in various nutrient media was assessed at weeks 1, 2 and 3 of cultivation using morphometric indicators such as shoot length in centimetres, average number of leaves in pieces, root length and the presence or absence of wilting and discoloration of leaves.

Statistical data was treated using methods of mathematical statistics with the calculation of the arithmetic average, its error, and the Fisher criterion in Microsoft Office Excel [29].

3 Results and discussion

As a result of experimental work in vitro, optimal sterilizing agents of *H. officinalis* were determined and data was obtained on the effect of the most effective sterilizing agent and its sterilization mode. The effect of sterilizing agents on obtaining sterile and viable explants of *H. officinalis* during seed sterilization, as well as sterilization modes are presented in Table 2 and Figure 1.

According to Fisher's exact criterion, at a significance level of $P > 0.05$, in terms of its effect on the number of sterile explants, all the obtained data presented in the table significantly differ from the control.

As it can be seen from table 2 and figure 1, the optimal sterilizing agents for *H. officinalis* seeds are a 5-15% solution of sodium hypochlorite with its effect for 10 minutes and a 10% solution of chloramine B with its effect for 10 minutes, since under these conditions the maximum ratio of the number of sterile (100%) explants

to the number of viable explants (100%) was obtained. It is also possible to sterilize seeds with 3% biocide for 30 minutes (86.0% viable explants), 0.1% silver nitrate with an exposure time of 10 minutes (80.33%). Sterilization with 10% chloramine B for 20 minutes, 5% chloramine B for 20 and 30 minutes and 5-15% sodium hypochlorite for 20 minutes gives the same yield of viable explants (60.67%). You can also use 5% lysoformin 3000 for 10 minutes (59.67% viable explants) and 5% biocide over time exposure time of 20 minutes (57.3% of viable explants). It is not advisable to use all other sterilization modes, since they produce less number of viable plant explants (less than 50%) or have a detrimental effect on the seeds.

In the course of the work the selection of the optimal composition of nutrient media was carried out to obtain mini plants of *H. officinalis* L., and callus cultures in vitro.

Plant sprouts of *H. officinalis* were cultivated on different media (the compositions are described in the research methods). The obtained parameters of the growth and development of mini-plants are presented in table 3, where SL is the shoot length, NL is the number of leaves, DR is the darkening of the root, CP is the color of the plant.

Based on the obtained data, the optimal medium for obtaining mini plants of the *H. officinalis* species is MS₅ medium, where there are more leaves, a more developed root system, and, in general, a more viable state of plants compared to other media. Optimum media for the induction of callus genesis are MS₄ and MS₆.

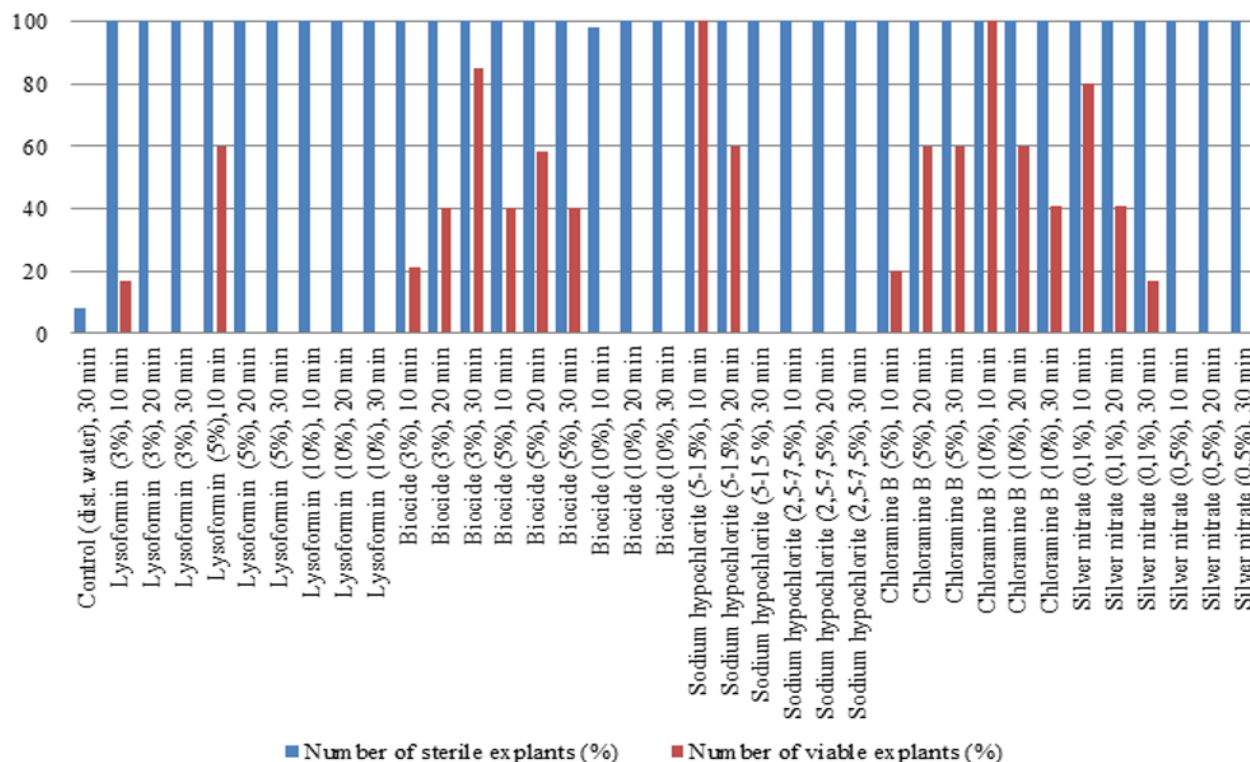

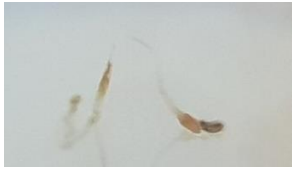
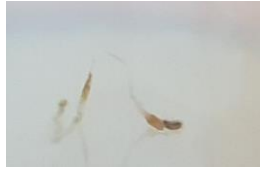























Fig 1. The effect of sterilizing agents on the number of sterile and viable explants of *H. officinalis*

Table 2. The effect of sterilizing solutions on plant explants *H. officinalis*

Sterilizing solution and its concentration	Sterilization duration (minutes)	Sterile explants number (%)	Viable explants number (%)
Lysoformin 3000 (3%)	10	99.67±0.11	14.67±0.11
Lysoformin 3000 (3%)	20	100.0±0.0	0.0±0.0
Lysoformin 3000 (3%)	30	100.0±0.0	0.0±0.0
Lysoformin 3000 (5%)	10	100.0±0.0	59.67±0.11
Lysoformin 3000 (5%)	20	100.0±0.0	0.0±0.0
Lysoformin 3000 (5%)	30	100.0±0.0	0.0±0.0
Lysoformin 3000 (10%)	10	100.0±0.0	0.0±0.0
Lysoformin 3000 (10%)	20	100.0±0.0	0.0±0.0
Lysoformin 3000 (10%)	30	100.0±0.0	0.0±0.0
Biocide (3%)	10	100.0±0.0	21.0±0.2
Biocide (3%)	20	100.0±0.0	40.33±0.11
Biocide (3%)	30	100.0±0.0	86.0±0.2
Biocide (5%)	10	100.0±0.0	40.33±0.11
Biocide (5%)	20	100.0±0.0	57.3±2.3
Biocide (5%)	30	100.0±0.0	41.0±0.2
Biocide (10%)	10	98.0±0.2	0.0±0.0
Biocide (10%)	20	100.0±0.0	0.0±0.0
Biocide (10%)	30	100.0±0.0	0.0±0.0
Sodium hypochlorite (5-15%)	10	100.0±0.0	100.0±0.0
Sodium hypochlorite (5-15%)	20	100.0±0.0	60.67±0.21
Sodium hypochlorite (5-15%)	30	100.0±0.0	0.0±0.0
Sodium hypochlorite (2,5-7,5%)	10	100.0±0.0	0.0±0.0
Sodium hypochlorite (2,5-7,5%)	20	100.0±0.0	0.0±0.0
Sodium hypochlorite (2,5-7,5%)	30	100.0±0.0	0.0±0.0
Chloramine B (5%)	10	100.0±0.0	20.33±0.11
Chloramine B (5%)	20	100.0±0.0	60.67±0.21
Chloramine B (5%)	30	100.0±0.0	60.67±0.21
Chloramine B (10%)	10	100.0±0.0	100.0±0.0
Chloramine B (10%)	20	100.0±0.0	60.67±0.21
Chloramine B (10%)	30	100.0±0.0	41.0±0.2
Silver nitrate (0,1%)	10	100.0±0.0	80.33±0.11
Silver nitrate (0,1%)	20	100.0±0.0	41.0±0.2
Silver nitrate (0,1%)	30	100.0±0.0	14.67±0.11
Silver nitrate (0,5%)	10	100.0±0.0	0.0±0.0
Silver nitrate (0,5%)	20	100.0±0.0	0.0±0.0
Silver nitrate (0,5%)	30	100.0±0.0	0.0±0.0
Control (distilled water)	30	6.0±0.2	0.0±0.0

TABLE 3. Cuitivation of *H. officinalis* on different types of culture media in invitro

Medium	Plant characteristics		
	Week 1 of cultivation	Week 2 of cultivation	Week 3 of cultivation
MS ₁	SL – 2,4 cm; NL –2; DR is not visible; CP – light brown  <i>H. officinalis</i> on MS ₁ medium	SL –2,4 cm; NL –2; DR is not visible; CP – intense brown  <i>H. officinalis</i> on MS ₁ medium	SL – 2,4 cm; NL –2; DR is not visible; CP – brown; the plant died  <i>H. officinalis</i> on MS ₁ medium
MS ₂	SL – 1,5 cm; NL –4; DR is visible; CP – intense green  <i>H. officinalis</i> on MS ₂ medium	SL – 1,9 cm; NL –4; darkening and redness of the root can be seen; CP – green  <i>H. officinalis</i> on MS ₂ medium	SL – 3,6 cm; NL –6; DR is visible; CP – green-brown  <i>H. officinalis</i> on MS ₂ medium
MS ₃	SL – 1,8 cm; NL –4; DR is not visible ; CP – intense green  <i>H. officinalis</i> on MS ₃ medium	SL – 2 cm; NL –4; DR is not visible; CP – intense green  <i>H. officinalis</i> on MS ₃ medium	SL – 2,7 cm; NL –6; DR is visible; CP – intense green  <i>H. officinalis</i> on MS ₃ medium
MS ₄	SL – 0,7 cm; NL –2; DR is not visible; CP – green  <i>H. officinalis</i> on MS ₄ medium	SL – 1,2 cm; NL –3; DR is not visible; CP – green with darkening  <i>H. officinalis</i> on MS ₄ medium	SL – 4,5 cm; NL –6; DR is not visible; CP – green; callus is formed.  <i>H. officinalis</i> on MS ₄ medium
MS ₅	SL – 1,1 cm; NL –4; DR is not visible; CP – green  <i>H. officinalis</i> on MS ₅ medium	SL – 2,2 cm; NL –4; DR is not visible; CP – green  <i>H. officinalis</i> on MS ₅ medium	SL – 2,3 cm; NL –9; DR is visible; CP – intense green  <i>H. officinalis</i> on MS ₅ medium

MS ₆	SL – 0,6 cm; NL –2; DR is not visible; CP – yellow-brown  <i>H. officinalis</i> on MS ₆ medium	SL – 0,6 cm; NL –2; DR is not visible; CP – yellow-brown  <i>H. officinalis</i> on MS ₆ medium	SL – 0,6 cm; NL–4; DR is visible; CP – green, callus is formed  <i>H. officinalis</i> on MS ₆ medium
MS ₇	SL – 1,8 cm; NL –2; DR is visible; CP – light-brown - green  <i>H. officinalis</i> on MS ₇ medium	SL – 1,8 cm; NL –2; DR is visible ; CP – white-brown  <i>H. officinalis</i> on MS ₇ medium	SL – 1,8 cm; NL –2; DR is visible; CP – brown – white-brown; the plant died  <i>H. officinalis</i> on MS ₇ medium
MS ₈	SL – 1,3 cm; NL –2; DR is visible ; CP – intense green  <i>H. officinalis</i> on MS ₈ medium	SL – 1,5 cm ; NL –4; DR is visible ; CP – intense green  <i>H. officinalis</i> on MS ₈ medium	SL – 1,6 cm; NL –4; DR is visible ; CP – intense green;  <i>H. officinalis</i> on MS ₈ medium

4 Conclusion

During the study, the introduction of *H. officinalis* plant explants into the in vitro culture was carried out, the sterilization process of *H. officinalis* plant explants was optimized, some of the most effective seed sterilization modes, their exposure time and concentration were found. The most optimal are 5-15% solution of sodium hypochlorite and 10% solution of chloramine B with its effect for 10 minutes, providing 100% sterilization of plant explants and most importantly 100% germination of viable seedlings.

Eight culture media were tested for the cultivation of *H. officinalis* seedlings. It was found that the most optimal nutrient medium for the induction of callusogenesis was a medium with indolyl-3-butyric acid - 1 mg / l, and a medium with α -naphthylacetic acid - 0.5 mg / l, kinetin - 0.1 mg / l and 6 -benzylaminopurine - 1 mg / l, and for the cultivation of plants *H. officinalis* in vitro - a medium containing indolyl-3-acetic acid IAA (2 mg / l), and kinetin (0.2 mg / l).

Thus, callus tissues and mini-plants of *H. officinalis* were obtained in vitro and the most optimal nutrient media were determined both for microclonal propagation and for the induction of callus tissue *H. officinalis*, which can be further used for mass cultivation of cell and culture and production from it safe bioadditives with active substances for livestock and crop production as part of the development of modern agrobiotechnology.

Acknowledge

This work was carried out as part of a state task FZWG-2020-0021.

References

- 1 S. V. Sizonenko and T. A. Belova, “*Hyssopus officinalis* to the culture in Medicinal plant growing: from the experience of the past to the latest technologies: materials of the International scientific and practical Internet conference, Poltava, 2012.
- 2 V. E. Torikov, “Medicinal value of vegetable, fruit and berry, field plants and wild plants,” (Publishing house of the Bryansk state agricultural Academy, Bryansk, 2013)
- 3 K. S. Timchuk, T. G. Zheleznyak, Z. N. Vorniku, and I. P. Dragalin, “Medicinal Hyssop - the content of essential oil and its component composition,” Materials of the international scientific conference (may 29-31, 2013, Minsk)
- 4 O. A. Grebennikova, A. E. Paliy, L. A. Khlypenko, V. D. Rabotyagov, Journal "Orbital" **1**, 1 (2017)
- 5 F. Fathiazad, S. Hamedeyazdan, Afr. J. Pharm. Pharmacol **5(17)**, 1959 (2011)
- 6 G. Zawiślak, Mod. Phytomorphol. **4**, 93 (2013)
- 7 A. Srivastava, K. Awasthi, B. Kumar, A. Misra, S. Srivastava, India Pharmacogn J. **10(4)**, 690 (2018)
- 8 L. A. Kotyuk, Biologichni Studii **7(2)**, 83 (2013)

- 9 A. N. Shibko and Yu. Aksenov, Ecosystems, their optimization and protection, **4**, 127-133 (2011).
- 10 A. Wesołowska, D. Jadcak, M. Grzeszczuk, Herba Pol. **56(1)**, 57 (2010)
- 11 V. Mitić, S. Đorđević, FU Phys. Chem. Tech. **2(2)**, 105 (2000)
- 12 M. Moghtader, J. Hortic. For. **6(1)**, 1 (2014)
- 13 H. Soleimani, M. Barzegar, M. A. Sahari, B. H. Naghdi J. of Med. Plants, **10(37)**, 61 (2011)
- 14 G. Schulz, E. Stahl, Flavor Fragr. J. **6(1)**, 69 (1991)
- 15 N. Dehghanzadeh, S. Ketabchi, A. Alizadeh, Asian J. Exp. Biol. Sci. **3(4)**, 767 (2012)
- 16 M. Jankovský, T. Landa, Hort. Sci. (Prague), **29(3)**, 119 (2002)
- 17 F. Fathiazad, M. Mazandarani, S. Hamedeyazdan, Adv. Pharm. Bull. **1(2)**, 63 (2011)
- 18 M. A. Myadelets, D. V. Domrachev, V. A. Cheremushkina, Chemistry of plant raw materials **1**, 111 (2012)
- 19 A. L. Budantsev and E. E. Lesiovskaya, Wild useful plants of Russia (SPKHFA Publishing house, Saint Petersburg, 2001)
- 20 D. Tančinová, Z. Mašková, D. Foltinová, J. Štefániková, J. Árvay, Potr. S. J. F. Sci. **12(1)**, 491 (2018)
- 21 T. G. Kuchina, I. K. Lebedev, and E. A. Kalashnikova, *Study of medicinal hyssop (Hyssopus officinalis L.) in culture in vitro* modern technologies in the world scientific space collection of articles of the International scientific and practical conference (September 28, 2017, Ufa).
- 22 B. Hosseini, M. Alizadeh, A. Hassani, M. Jafari, A. Rahimi, J. Medicinal Plants By-products **2**, 187 (2016)
- 23 E. Zayova, M. Geneva, I. Stancheva, L. Dimitrova, M. Petrova, M. Hristozkova, I. Salamon, Med. Plants, **10(4)**, 295 (2018)
- 24 I. Stancheva, M. Geneva, M. Hristozkova, E. Zayova, J. Herbs Spices Med. Plants **25(2)**, 104 2019
- 25 A.V. Kilchevsky, Genetic bases of plant breeding: Biotechnology in plant breeding. Cell Engineering. (Nauka, Minsk, 2012).
- 26 T. Murashige, Physiol Plant **15(3)**, 473 (1962)
- 27 V. S. Shevelukha, Agricultural Biotechnology. (Vyshaya Shkola, Moscow, 2008)
- 28 O. A. Timofeeva and N. I Rummyantseva, Culture of plant cells and tissues (Kazan University, Kazan, 2012)
- 29 Pifo Hans-Peter. Statistics.(Ed. VNIIA, Moscow, 2011)