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Optimizing in vitro introduction of wild common hop (*Humulus lupulus* L.) specimens using plant hormones

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Abstract

Hop (*Humulus lupulus* L.) is a perennial crop propagated vegetatively. There are a number of problems associated with preserving the gene pool, obtaining certified planting material, and replenishing the collection of hop varieties. To solve these problems, it is promising to use biotechnological methods and attract genetic resources from natural flora. The article presents the results of an experimental research on introducing into tissue culture and studying the effect of cytokinin growth regulators on in vitro reproduction of wild hop genotypes from the Altai Krai. A comparison with the in vitro morphogenesis of varieties bred in Russia is provided. Wild hop samples have been shown to be successfully introduced into tissue culture at a level not lower than that of national varieties and are characterized by intensive growth of shoots and root systems in hormone-free Murashige and Skoog (MS) medium. The ANOVA results confirmed that the genetic characteristics of the samples had the greatest impact on the reproduction rate and height of hop plants in vitro culture. The second most important factor was the type of plant growth regulator. The maximum height of the shoots of the 'Flagman' variety and the wild sample AK-3 was in the MS nutrient medium containing 5 μM kinetin, and for the 'Civil'skij' variety – 1 μM 6-benzylaminopurine. For the AK-1 genotype, the best results were obtained in hormone-free MS medium. The reproduction rate, calculated as the ratio of the height of shoots, including the lateral ones, to the number of internodes, ranged from 3.9 to 9.1. For wild samples, it was possible to achieve reproduction rates of 8–9 in one passage lasting 8 weeks. The results confirmed the high morphogenetic potential of wild hop samples AK-1 and AK-3 from the south of Western Siberia, as well as the national variety 'Flagman' for the use of clonal micropropagation technology to preserve genotypes in the in vitro collection, breeding, and production of planting material.

Keywords

Clonal micropropagation, hops (*Humulus lupulus* L.), morphogenesis, plant growth regulators, south of Western Siberia

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Introduction

Mobilization, conservation, and use of crop genetic resources, including their wild relatives, are of strategic importance for breeding new varieties and ensuring sustainable crop production (Girichev et al. 2012; Tyagi and Grawal 2015). The most common method of long-term ex situ preservation is dry and cool storage of seeds, which tolerate severe dehydration and low temperatures. However, in some cases, seed production is not possible due to sterility or segregation of highly heterozygous genotypes (Rajasekharan and Sahijram 2015). Typically, such crops are propagated vegetatively and each genotype must be maintained as a clone. For these accessions, there are two main conservation approaches: i) conservation of plants in field collections/genbanks; ii) establishment of a cell and tissue culture bank (i.e., in vitro storage) in the form of actively or slowly growing collections or under cryopreservation conditions (Mitrofanova et al. 2018; Panis et al. 2020). A field gene bank is a collection of living plants that remain in one place for many years, making it vulnerable to a number of negative factors related to pests, diseases, and environmental conditions. Maintaining such a bank is expensive and, unlike other forms of storage, requires constant care. In vitro storage has significant potential in this situation. In addition, biotechnological methods make it possible to eliminate viral, viroid, and bacterial infections from valuable genotypes and to speed up the process of obtaining high-quality planting material.

Common hop (*Humulus lupulus* L.) is a perennial dioecious climbing vine, widespread in the temperate climates of Eurasia and North America, and belongs to vegetatively propagated crops. Female plants are grown in high trellises, in one place for 10–15 years, which is a threat to the phytosanitary condition of the plants. Hop strobili (*cones*) are used primarily as bittering, flavoring, and stabilizing agents in beer. In addition to bitterness, they give a floral, fruity, or citrus flavor and aroma. Recent studies have shown that hops contain dozens of secondary metabolites that have potential pharmacological and medicinal value. For example, they are promising in the development of antimicrobial drugs, cancer and metabolic syndrome treatments, as well as insecticides, preservatives and flavors (Korpelainen and Pietiläinen 2021; Pereira et al. 2022).

Currently, there are several hundred varieties of hops in the world, and new varieties are being developed and tested. In Russia, over the past decades, the situation with both the production of hop products for brewing and the breeding and nursery farming of hops remains complex (Khlynovskiy et al. 2023). As of 2023, only 13 varieties of hops of national breeding were included in the State Register of Breeding Achievements Approved for Use, the originator of which is the Federal Agricultural Research Center of the North-East named after N.V. Rudnitsky. Based on a branch of this organization – the Chuvash Research Institute of Agriculture, the only field genetic collection of hops in the country has been created and maintained, numbering 250 samples of wild and varietal hops from various regions of Russia and 17 foreign countries (Nikonova, Korotkova, 2017). The problems of increasing the diversity of national varieties and mass production of planting material can be solved by creating in vitro collections and involving in breeding programs the genetic resources of wild hop populations, which are distinguished by significant diversity in the south of Western Siberia. To create a tissue culture bank, the key element is the selection of effective protocols to introduce explants into aseptic culture and to determine the composition of nutrient medium for specific genotypes. The regulatory mechanism of organogenesis and embryogenesis is the balance of plant growth regulators, usually auxins and cytokinins (Yamaguchi et al. 2010). Previous studies have shown significant variability in the response of hop varieties in cell and tissue culture, from variants with high reproduction rates to cases where in vitro cultivation is impossible after the first couple of passages (Kastritskaya et al. 2014; Gashenko et al. 2019). The purpose of the study was to investigate the effect of cytokinin growth regulators on in vitro reproduction of wild hop genotypes and to compare the morphogenesis of the samples with varieties of national breeding.

Materials and methods

The studies were conducted at the Altai Center for Applied Biotechnology of the Altai State University (Barnaul). The objects were 4 genotypes of *H. lupulus*: two wild samples (AK-1, AK-3) collected in the south of western Siberia in the Altai Krai and two varieties (‘Civil'skij’, ‘Flagman’) from the field collection of the Chuvashia Research Institute of Agriculture. The wild samples attracted attention for their morphometric characteristics. These are a powerful bush, abundant fruiting, large dense ‘cones’, well-developed lupulin grains, a pleasant rich aroma. They were classified as promising forms for involvement in the breeding process. Civilskij is an early-ripening variety of the aromatic type, the growing season lasts 95–100 days, the technical ripeness is short, which requires harvesting in a limited time. The average yield is 2.25 t ha^{-1}, the alpha acid content is 5.5%. The mid-early ‘Flagman’ variety is a sample of bitter-aromatic type, the growing season is

110–112 days. The average cone yield is 3.8 t ha^{-1}, the alpha acid content is 6.5%. Both varieties are actively used in brewing in Russia. According to FAO standards (FAO. 2014. Genebank…), precise field data were recorded, including geographical coordinates determined using a GPS navigator, for wild samples. Control herbarium specimens, as well as photographs of habitus and habitat, are stored at Altai State University (Fig. 1).

Figure. 1. Plant appearance in nature and herbarium sample of *Humulus lupulus*

The initial hop material for introduction into in vitro culture was grown under artificial climate conditions in the greenhouse to reduce the potential infection of future explants with fungal and bacterial spores and to ensure year-round availability of samples for work. The stem and rhizome cuttings taken from the plantation and in nature were planted on 3-4 liter containers in the soil substrate. Two-bud cuttings (nodal segments) were isolated from actively vegetating shoots and used as explants.

At the stage of introduction to the in vitro culture, a medium with a mineral composition according to the Murashige-Skoog (MS) recipe (Murashige and Skoog 1962) was used, supplemented with 20.0 $g{\cdot}L^{-1}$ glucose, 7.3 $g{\cdot}L^{-1}$ agar-agar, 2.0 $mg{\cdot}L^{-1}$ 6-benzylaminopurine (BAP) and 1.0 $mg{\cdot}L^{-1}$ gibberellic acid (GA). At the shoot multiplication stage, BAP and kinetin (KIN) were used as cytokinin growth regulators at concentrations of 0.5, 1.0, 1.5, 3.0, and 5.0 μMol. The PGR-free MS medium served as a control. Fifteen explants/cuttings were used for each variant.

To initiate the primary sterile culture, the following nodal segment sterilization was used: soap solution (15 min) running water (60 min) → 70% ethyl alcohol (1 min) → 30% hydrogen peroxide + 2 drops of Twin 20 (10 min) → three rinses with sterile water (× 5 min). The culture was carried out on racks under standard conditions at a temperature of 21 to 23 ° C, 16 hours of daylight, and illumination of 3000 lux. Glass tubes measuring 21 × 200 mm with 10 ml of nutrient medium were used as culture vessels. Three weeks after planting the nodal segments, the number of infected, necrotic, and viable explants from the total number was taken into account. At the multiplication stage, after 8 weeks of cultivation, the number and length of the shoots, the number of internodes, the presence and size of the callus, the development of the root system, the presence of visually distinguishable deformations and deviations were observed. The reproduction rate (RR) was calculated as the ratio of the height of shoots, including lateral ones, to the number of nodal segments. Statistical processing of the experimental data was performed using Microsoft Office Excel 2016 and Statistica 10.

Results and discussion

One of the most important components of the stage of introducing plants into in vitro culture is the use of a sterilization scheme, which, with effective decontamination of explants, should not damage plant tissues and affect their ability to continue biological development in vitro culture (Demidchik et al. 2019). The sterilization protocol allowed us to obtain an average of 79.4% sterile explants in the four samples. However, the number of infected explants varied significantly between individual genotypes (Table 1). The difference between the wild and varietal samples was more than 2.6 times.

The number of viable sterile explants also differed by more than 35.4% depending on the plant genotype. The sterilization scheme was most traumatic for varieties, where on average 39.2% of the sterile explants turned out to be necrotic. The maximum proportions of viable explants were found in wild hop samples AK-3 and AK-1 and averaged 90.3% of the sterile ones and 80.9% of the introduced ones. The worst result of introduction into in vitro culture compared to other genotypes was shown by

the ‘Civil’skij variety, whose microcuttings were the most infected and were more frequently subjected to necrosis. Thus, the selected sterilization regime turned out to be quite effective, especially for wild hop samples. Explants after sterilization were highly viable, allowing us to obtain a well-growing sterile culture.

Table 1. Efficiency of sterilization of hop explants

Genotype	Number of explants						
	total, pcs.	infected		necrotic*		viable*	
		pcs.	%	pcs.	%	pcs.	%
AK-1	52	10	19.2	6	14.3	36	85.7
AK-3	81	3	3.7	3	3.8	75	94.9
‘Civil'skij’	75	33	44.0	17	40.5	25	59.5
‘Flagman’	169	26	15.4	54	37.8	89	62.2

Note: * – from the number of sterile explants

The number of viable sterile explants also differed by more than 35.4% depending on the plant genotype. The sterilization scheme was most traumatic for varieties, where on average 39.2% of the sterile explants turned out to be necrotic. The maximum proportions of viable explants were found in wild hop samples AK-3 and AK-1 and averaged 90.3% of the sterile ones and 80.9% of the introduced ones. The worst result of introduction into in vitro culture compared to other genotypes was shown by the ‘Civil’skij variety, whose microcuttings were the most infected and were more frequently subjected to necrosis. Thus, the selected sterilization regime turned out to be quite effective, especially for wild hop samples. Explants after sterilization were highly viable, allowing us to obtain a well-growing sterile culture. Plant morphogenesis in vitro depends on internal and external factors. The first group of factors includes the species of the source material, the genotypic characteristics of the plants, the age of the mother plant, the organ from which the explant was taken, etc. Significant external factors are the cultivation conditions and, above all, the hormonal composition of the nutrient medium (Kosenko et al. 2021; de Souza et al. 2022). There is evidence that the PGR-free nutrient medium was optimal for some hop samples (Machado et al. 2018; Hirakawa and Tanno 2022), while other authors (Roy et al. 2001; de Souza et al. 2022; Iacuzzi et al., 2023) suggested the use of BAP, GA, thidiazuron (TDZ), either separately or in combination with each other or auxins, to improve the efficiency of in vitro propagation.

In this work, the sterile plants at the shoot multiplication stage were propagated by cuttings and transferred to nutrient media supplemented with cytokinins. The samples differed both in growth dynamics and in response to plant hormones (Fig. 2). Depending on the genotype, the shoot height in the control in PGR-free MS medium varied from 21.7 mm (‘Civil'skij’) to 132.4 mm (AK-1) and averaged 99.3 mm. Three genotypes showed similar growth rates and fluctuated insignificantly from 118.0 to 132.4 mm, and the ‘Civil'skij’ variety was characterized by weak development. At the same time, the ‘Civil'skij’ variety turned out to be more responsive to enrichment of the nutrient medium with growth regulators. Only in this genotype, of 10 variants of the experiment with BAP and KIN, 9 showed an excess of control values. The average shoot height was 51.8 mm (Fig. 2A). The maximum height of the main shoot (69.3 mm) was obtained using 1 μM KIN, which was 3.2 times higher than the control value. The fast-growing wild hop genotypes and the ‘Flagman’ variety responded differently to both the type of cytokinin and its concentration. The ‘Flagman’ variety showed positive dynamics compared to the control only in one variant of the experiment with KIN, and in the BAP it had the smallest height of the main shoot (Fig. 2B). The AK-3 sample was characterized by a variable response to exogenous hormones. In 6 variants, the shoot height exceeded the control by 0.8–45.8 mm, which amounted to 0.7–38.8%. The use of KIN at the maximum concentration (5 μM) allowed increasing the trait in the‘Flagman’ variety to 139 mm, and in the sample AK-3 by 1.4 times relative to the control, which were the maximum values for both genotypes (Fig. 3). When using KIN, hop regenerants developed leaf blades without signs of anomalies, the root system was well formed, and the plants did not show signs of vitrification. The AK-1 showed negative dynamics for cytokinins in all variants.

In hops, root formation in vitro occurs in parallel with shoot formation. After 8 weeks of cultivation, the root length of all samples averaged 18.3 mm (Fig. 4). It was observed that the root system developed regardless of the presence and concentration of hormones in the nutrient medium. Maximum values, which exceeded control by more than 1.8 times, were observed for the AK-1 and ‘Civil'skij’ samples in KIN-free medium at a concentration of 1 μM.

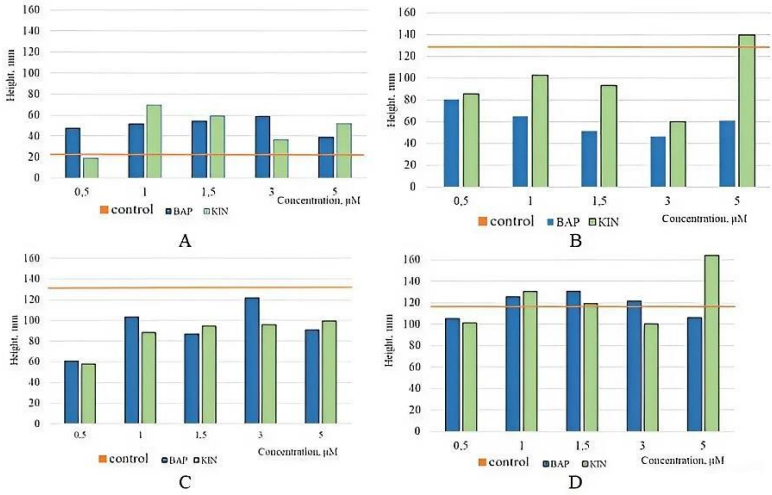

Figure 2. Height of the main shoot of hops in media supplemented with cytokinins: A – ‘Civil'skij’ B – ‘Flagman’; C –AK-1; D – AK-3

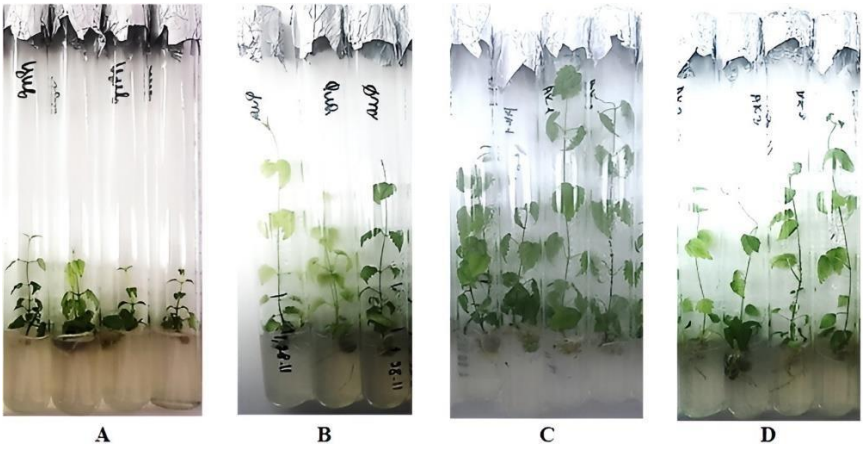

Figure 3. Hop regenerants on a medium containing 5 µM KIN: A – ‘Civil'skij’ B – ‘Flagman’; C – AK-1; D –AK-3

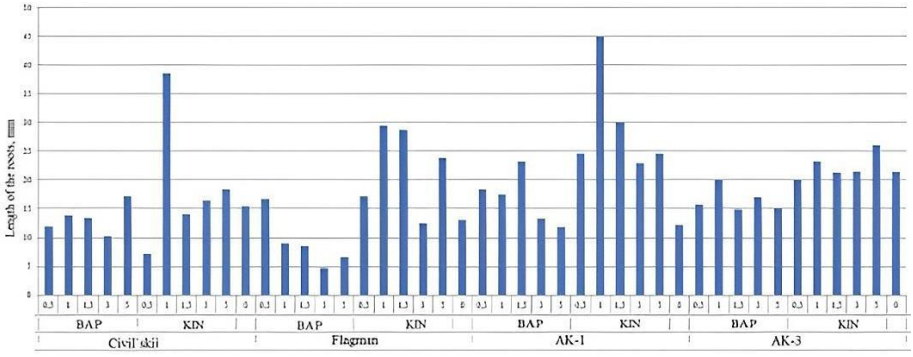

Figure 4. Average length of hop roots depending on the type and concentration of plant growth regulator

In some variants, excessive callus formation was observed, which had a negative effect on root development. Callus formation occurred mainly in BAP-containing media. With an increase in the concentration of this hormone, the proportion of shoots forming callus also increased (Fig. 5).

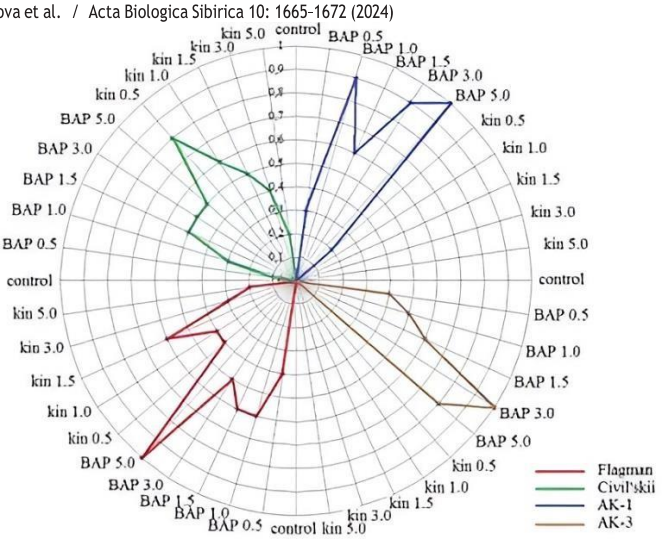

Figure 5. The proportion of hop regenerants with developed callus depends on the type and concentration of plant growth regulator.

The loose non-morphogenic callus resulted in poor root system. Roots, compared to the control, were few in number, thin, and unbranched. In some cases, vitrification and development anomalies were observed (Fig. 6).

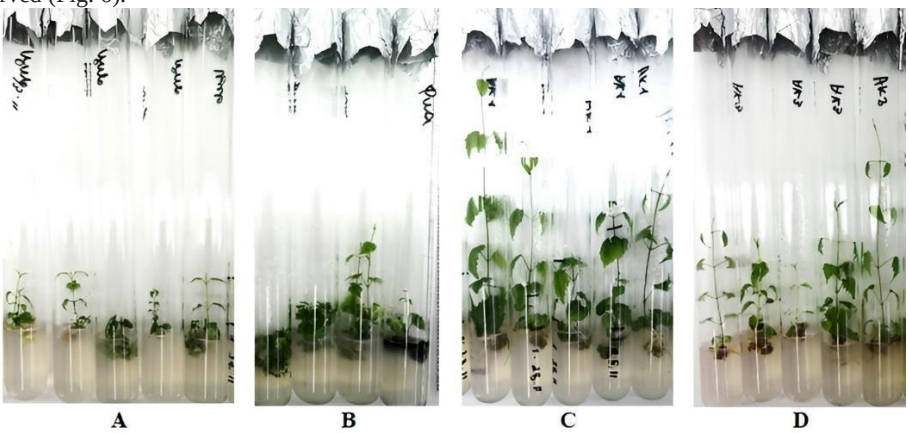

Figure 6. Hop regenerants on a nutrient medium containing 5 µM BAP: A – ‘Civil'skij’ B – ‘Flagman’; C – AK-1; D –AK-3

An important parameter that determines the success of the shoot multiplication stage is the reproduction rate. The higher the RR during cultivation, the more plant material can be obtained per unit of time. In plants with rapid vertical growth in vitro culture (hops, grapes, potatoes, etc.), the increase in the reproduction rate can occur either due to an increase in the number of nodal segments or due to the development of additional shoots from axillary buds. Most often, cytokinins are used to remove apical dominance and induce the development of axillary buds in many plant species (Shimizu-Sato et al. 2009). The reproduction rate, calculated as the ratio of shoot height, including lateral ones, to the number of nodal segments, ranged from 3.9 to 9.1 (Table S2). For wild hop samples AK-1, AK-3, and the ‘Flagman’ variety, the reproduction rates in the control were 7.5 to 7.6 and did not differ statistically when the medium was enriched with growth regulators, regardless of the type and concentration of the hormone. Only in the ‘Civil’skij variety did cytokinins provide a significant increase in the reproduction rate compared to the control, which was 4.4. Enrichment of the nutrient medium with BAP at a concentration of 3 µM increased RR to 7.0. The addition of KIN to the medium at concentrations of 1.0 and 1.5 µM increased RR to 7.5 and 7.7, respectively.

To identify factors that had a significant impact on the main indicators of hop clonal

micropropagation, a three-way ANOVA was carried out (Table S3). The proportion of factors influence of such as “genotype”, “hormone type”, “hormone concentration”, as well as their interaction were calculated. The ‘genotype’ factor was found to contribute significantly to the variability of reproduction rate and plant height, accounting for 37 and 71%, respectively. Numerous experiments by national and foreign researchers also confirmed the important role of genotype in controlling the growth and morphogenesis processes of various crops in vitro (Pua and Gong 2004; Molokanova et al. 2018; Krakhmaleva et al. 2019; Lutova and Dodueva 2019; Shulgina et al. 2021; Bedir et al. 2022). Scientists noted the importance of taking into account the origin of genotypes when developing in vitro culture protocols. For example, hop genotypes of American origin are less efficient in phytohormone synthesis compared to genotypes of European origin, resulting in an increased morphogenetic potential of the latter (de-Souza et al. 2022). The hormonal composition of the nutrient medium, as noted above, is the second key factor in the cultivation of cells and tissues. The ‘hormonal type’ factor significantly influenced the reproduction rate, equating to 30%.

Cytokinins are known to induce the development of axillary buds in hemp, hops, and other plant species (Romanov and Medvedev 2006; Lata et al. 2009), while simultaneously suppressing shoot elongation (Sugla et al. 2007). In the experiments, the height of the main shoot depended more on the concentration of the growth regulator (5%) than on its type (3%). The factor made an insignificant contribution to the reproduction rate.

Conclusıon

The analysis of experimental data on the introduction of four hop genotypes into tissue culture allows us to draw the following conclusions. Wild hop samples were successfully introduced into tissue culture at a level not lower than that of national varieties and characterized in vitro by intensive growth of shoots and roots in PGR-free MS medium. The use of two-bud cuts (nodal segments) as explants and a sterilization protocol in which 70% ethyl alcohol (1 min exposure) and 30% hydrogen peroxide (10 min exposure) were used successively as sterilizing agents allowed us to obtain an aseptic hop culture. This sterilization protocol proved to be 35.7% more effective for wild samples compared to varietal ones. According to the results of a three-way ANOVA, it was proven that the genetic characteristics of the samples had the greatest impact on the reproduction rate and height of the hop plants. The type of hormone was the second most important factor. The maximum shoot height of the ‘Flagman’ variety and the wild sample AK-3 was observed in the MS nutrient medium containing 5 μM KIN, and for the ‘Civil'skij’ variety – 1 μM BAP. For the AK-1 genotype, the best results were obtained in hormone-free MS medium. The reproduction rate, calculated as the ratio of the height of shoots, including the lateral ones, to the number of nodal segments, ranged from 3.9 to 9.1. For wild samples, it was possible to achieve reproduction rates of 8–9 in one passage lasting 8 weeks.

The results confirmed the high morphogenetic potential of wild hop samples AK-1 and AK-3 from the south of Western Siberia, as well as the national variety ‘Flagman’ for the use of clonal micropropagation technology while preserving genotypes in the in vitro collection, breeding and production of planting material.

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