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The effect of growth regulators on the multiplication of *Crataegus sanguinea* *in vitro*

This study focused on examining how plant growth regulators affect shoot proliferation on *Crataegus sanguinea*. Shoot multiplication treatments included benzylaminopurine (6-BAP) at different concentrations (0.25, 0.5, and 0.75 mg/l) combined with constant 0.5 mg/l gibberellic acid (GA3) and 0.01 mg/l Indole-3-butyric acid (IBA), in *Quoirin & Lepoivre* (QL) nutrient medium. The results showed that the highest number of shoots per explant (5.23) was achieved in a medium supplemented with 0.5 mg/l 6-BAP plus 0.5 mg/l GA3 and 0.01 mg/l IBA, while the greatest shoot length (12,77 cm) was obtained with 0.75 mg/l 6-BAP plus 0.5 mg/l GA3, and 0.01 mg/l IBA 35 days after transplantation. This study presents an efficient protocol for *in vitro* multiplication of *Crataegus sanguinea*, achieving the highest shoot proliferation rate.

Keywords: Axillary buds, QL medium, PGRs, Shoot proliferation, *Crataegus sanguinea*.

Introduction

Sexual reproduction in plants significantly contributes to the diversity of reproductive strategies. Mechanisms such as self-incompatibility and dioecy facilitate cross-pollination, which enhances genetic variability (genetic recombination) and adaptability by increasing the potential for adaptive responses to environmental changes [1].

Crataegus spp., which belongs to the subfamily *Maloideae* within the *Rosaceae* family [2], exemplifies the complex interplay of these reproductive strategies [1]. This genus is particularly notable for its extensive hybridization capabilities, a consequence of its sexual reproduction mechanisms [2].

Hybridization complicates traditional taxonomic and genetic works by introducing a range of unpredictable outcomes and intermediate traits. This process affects ploidy levels, leading to additional challenges related to fertility and genetic stability. It can also influence gene flow between species, impacting their evolutionary paths and potentially contributing to speciation or extinction events [3]. This complexity is partly attributed to its base haploid chromosome number of x=17, which contributes to the genetic diversity and variability within the genus [2].

The genus includes both shrubby species and others that can reach heights of up to 12 meters. Widely distributed across the temperate regions of the Northern Hemisphere, *Crataegus* comprises approximately 280 species [2].

Hawthorn, a wild edible plant, has played a vital role in human life for centuries. Historically, its fruits, seeds, leaves, flowers, roots, and branches have been utilized to fulfill various personal and societal needs, including as a food source, for medicinal purposes, and as an ornamental plant [4].

Various *Crataegus* species are listed in the pharmacopeias of Germany, Britain, France, Switzerland, the US [5], Canada and China [6].

Due to their antispasmodic, cardiotonic, diuretic, hypotensive, and antiatherosclerotic properties. In Europe and the USA, aqueous ethanol extracts of *Crataegus* are used clinically to manage heart failure. These extracts are also employed to address a range of health issues, including cardiovascular disorders and concerns related to the central nervous system, immune system, eyes, reproductive system, liver, and kidneys [5]. Additionally, *Crataegus* extracts exhibit cytotoxic effects, gastroprotective properties, anti-inflammatory mechanisms, and antimicrobial activity [2].

The leaves, flowers, and fruits of *Crataegus* species are particularly rich in antioxidants [4], containing phenolic compounds such as chlorogenic acid, epicatechin, and hyperoside, which help to reduce genetic damage in bone marrow cells [7].

However, pharmaceutical products containing secondary metabolites must meet specific phytochemical content requirements, including minimum concentrations of active components like flavonoids and procyanidins [6]. All above-mentioned benefits highlight the plant's potential to contribute to overall human health and well-being.

Characterization of secondary metabolites through mass spectrometry or nuclear magnetic resonance (NMR) spectroscopy offers an additional method for describing *Crataegus* [8]. However, this kind of dataset alone does align with the evolutionary pattern of the species Molecular analysis based on ITS and 5.8S ribosomal DNA sequences shows that *Crataegus* species are not monophyletic [8].

In horticultural practice, *Crataegus* species are extensively utilized for their beneficial properties. These species are valued not only for their role in rootstock but also for the nutritional content of their fruits. The fruits are rich in sugars (4–11 %), pectin (0.6-1.6 %), tannins, and pigment compounds (0.8-1.7 %). They also have significant amounts of ascorbic acid (vitamin C) and vitamin A, with concentrations ranging from 31 to 108 mg and 380 to 680 mg per 100 grams, respectively [9].

In horticulture, *Crataegus* species are extensively utilized as rootstocks for apple, pear, and quince trees. The fruits of these species are characterized by their content of sugars (4–11 %), pectin (0.6-1.6 %), tannins, and pigment compounds (0.8-1.7 %). In addition, these fruits are distinguished by their significant content of ascorbic acid (vitamin C) and vitamin A, with concentrations varying between 31 and 108 mg, and 380 and 680 mg per 100 grams, respectively [9].

In Kazakhstan, seven wild species are found: *Crataegus almaatensis*, *Crataegus ambigua*, *Crataegus cholorocarpa*, *Crataegus pontica*, *Crataegus sang uinea*, *Crataegus songarica*, *Crataegus turkestanica*. *Crataegus ambigua* is listed in the Red Book of Kazakhstan [9].

Morphologically, the stems of *these* species are covered in thorny spines that 3–6 cm in length. Initially smooth, the bark is gray with light or dark tints. Mostly, flowers of wild types, typically white, have an unpleasant aroma. Cultivars of *Crataegus* sometimes produce pink or red flowers. The fruit is a small pome containing 1–5 seeds, and it can be orange-yellow, red-purple, or black in color [9].

Crataegus sanguinea are thorny shrubs or small trees, 1–4 meters tall, found in forest, forest-steppe, and steppe zones. They have purple-brown, glossy branches with strong, red-brown thorns. A hundred grams of fruits contain 127 mg of vitamin C [9]. In their leaves, various compounds have been found, including flavonoids (such as hyperoside, vitexin, apigenin, luteolin, quercetin, and rutin), phenylpropanoids (like caffeic acid and chlorogenic acid), saponins, vitamins, and various other secondary and primary metabolites, all of which are highly valued for their medicinal properties [10].

Hawthorn trees are winter-hardy, light-demanding, and drought-resistant, requiring little in terms of soil quality. They propagate naturally through seeds or sprouts, while in cultivation they can be propagated by layering or grafting. These trees have a lifespan of 200 to 300 years [9]. Typically, attempts to propagate hawthorns using standard horticultural techniques have encountered significant challenges. Seed germination tends to be slow and has a low success rate, while cuttings from mature trees are difficult to root and rarely succeed [11]. Currently, the *in vitro* culture method is extensively employed to address issues related to *ex situ* preservation and the restoration of the gene pool of rare and endangered plant species. This technique allows for the production of a larger quantity of plant material and produces disease-free plants [12]. Moreover, the approach focuses on maintaining genetic stability of meterial [1].

Developed protocols may serve as an excellent starting point. *In vitro* techniques have been applied to *Crataegus aronia* L. [13], *Crataegus oxyacantha* [14], *Crataegus pseudoheterophylla* Pojark [15], *Crataegus monogyna* [16].

Crataegus sanguinea, along with other *Crataegus* species, is recognized for its medicinal properties and horticultural value. However, there is limited research focused solely on *Crataegus sanguinea*, in the context of propagation.

In our study, we aim to develop an applicable protocol for the shoot multiplication of medically important species of *Crataegus sanguinea* for conservation and reproduction.

Experimental

Sample collection and explants preparation

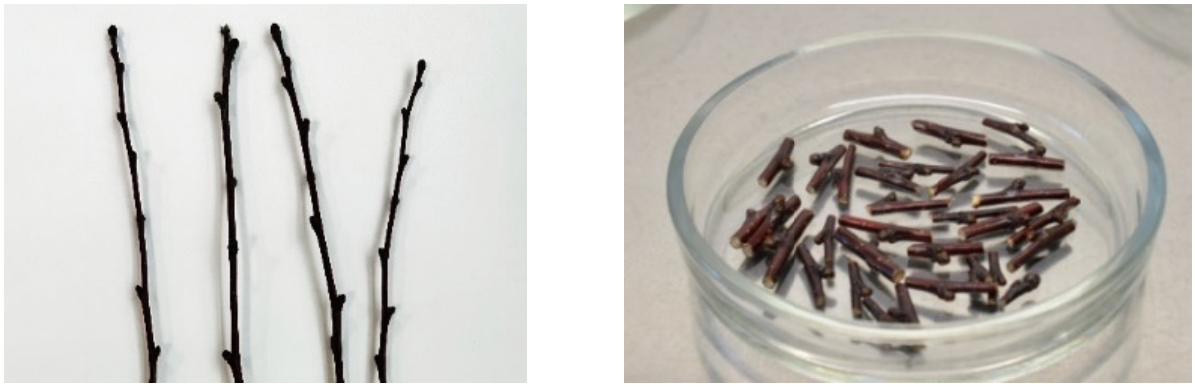
The stems of hawthorn were collected in 2023 from two regions, Karaganda (Korneevskiy Forests, Bukhar-Zhyrau District) and Aktau. The GPS data of the collected samples are given in Table 1. Axillary buds were stored at 4 °C until use.

Table 1

Coordinates of the collected plant materials

Species	Geographical location	Longitude	Latitude	Height above sea level, m
Crataegus sanguinea	"Korneevsky" forest, "Bukhar-Zhyrausky district, Karaganda region	E073°95.693'	N50°29.607'	552
	Aktau region	E51°9'39.337"	N43°39'1.969"	

Axillary buds (Fig. 1), excised from nodal segments, and 2-2.5 cm long segments were exposed to sterilization with soapy water three times and then washed under running tap water for 30 min. The effectiveness of hydrogen peroxide (H_2O_2) at various concentrations (5 %, 10 %, and 15 %) was studied for the establishment of *in vitro* culture, with sterilization duration of 5 minutes. Afterward, the explants were rinsed three times with sterile distilled water and dried on filter paper in Petri dishes for approximately an hour. The prepared axillary buds were cultured on QL medium to examine the viability of the donor plant.



A) Collected donor plants B) Prepared segments of plant material

Figure 1**.** Axillary buds of *Crataegus sanguinea*

Formation of the main shoot

After obtaining sterile and viable explants, the next stage was selecting the nutrient medium. The prepared axillary buds were cultured in QL, *Murashige and Skoog* (MS), and *Woody Plant Medium* (WPM).

In our experiment, nutrient mediums were supplemented with constant 0.25 mg/l 6-BAP and 0.5 mg/l GA3 to regenerate the main shoots. The number of shoots obtained at the sterilization and regeneration stages was recorded to calculate the percentage of viable explants.

Shoot multiplication

Regenerated shoots were cultured in glass jars containing QL medium, supplemented with 6-BAP at four concentrations in combination with constant IBA and GA3 for shoot multiplication. As a result, the following treatments were studied: I — PGR-free medium (control); II — 0.25 mg/l 6-BAP, 0.5 mg/l GA3, and 0. mg/l IBA in QL; III — 0.5 mg/l 6-BAP, 0.5 mg/l GA3, and 0.01 mg/l IBA in QL; IV — 0.75 mg/l 6-BAP, 0.5 mg/l GA3, and 0.01 mg/l IBA in QL. In our study, 30 explants were cultivated for each treatment, and data were collected 35 days after transplantation.

Statistical analyses involved subjecting the experimental results to ANOVA, with significant differences determined using Tukey's post hoc test in SPSS 25.0 (IBM Inc., New York, NY). The data represent means ± standard error from three independent experiments.

The cultures were incubated in a climate chamber at 24–26 °C, with a relative humidity of 60–80 %. The day length was maintained at 16 hours in a 24-hour light/dark cycle.

Results and Discussion

Culture establishment

The success of in vitro culture largely depends on several factors, including the genetic potential of the explants, their morphogenetic stages of development, and the establishment of aseptic conditions that ensure higher survival rates and low percentage of contamination. Therefore, selecting an effective (non-residual) sterilization agent is a crucial step [17].

Numerous studies indicate that H_2O_2 is a suitable sterilization agent for certain species in the Rosaceae family. By using the optimal concentration, H_2O_2 can effectively sterilize axillary buds, ensuring high explant viability while minimizing the risks of necrosis and contamination [18].

Based on the significant research findings of previous studies, our research tested different concentrations of H_2O_2 for sterilizing Crataegus explants. The 10 % H_2O_2 solution achieved the highest viability, with 73,3 % of hawthorn explants remaining sterile (22 explants out of 30).

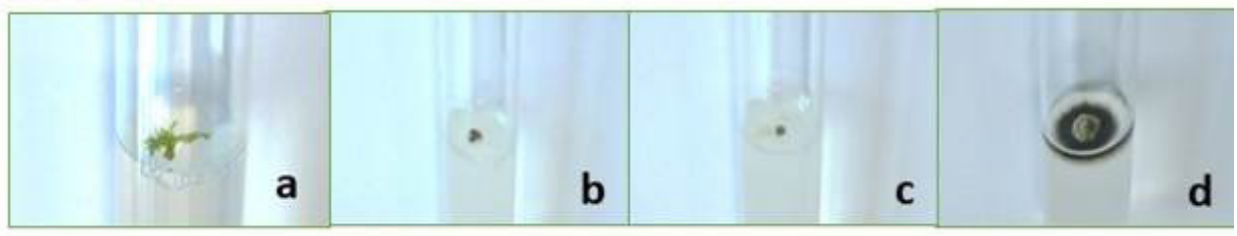
In contrast, the 5 % H_2O_2 solution resulted in a high infection rate of 86,6 % (26 explants out of 30) and lower viability 13.3 % (4 explants out of 30). The use of a 15 % H_2O_2 solution resulted in significant explant necrosis, affecting up to 66.6 % of the samples (Table 2).

Table 2

Results of sterilization of hawthorn (*Crataegus sanguinea*) explants

Treatment	Explant contamination		Explant necrosis		Viable explants	
	pcs.	%	pcs.	%	pcs.	%
I — 5 % H_2O_2	26	86.6	0	0	4	13.3
II — 10 % H_2O_2	6	20.0	2	6.6	22	73.3
III — 15 % H_2O_2	0	0	20	66.6	10	33.3

Therefore, the optimal concentration for axillary buds of *Crataegus sanguinea* is a 10 % H_2O2 solution (Fig. 2).



A) Sterile and viable explants B) Explant necrosis C, D) Explant contamination

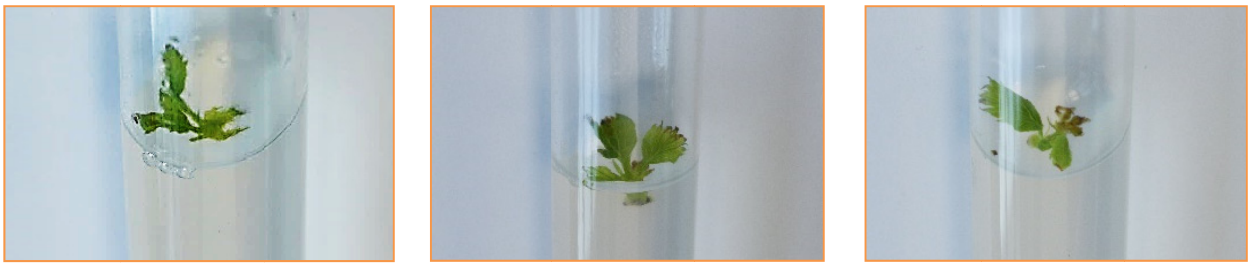
Figure 2. Sterilization of axillary buds of *Crataegus sanguinea*

Initial shoot formation

The initial shoot formation is one of the critical stages that determine the success of plant tissue culture. A suitable nutrient medium, proper balance and concentration of PGRs are essential for stimulating the explants to develop into shoots.

Successful shoot initiation of *Crataegus* species on MS, LP and WPM has been published. It was reported that the optimal concentration of growth regulators, specifically, 6-BAP and IBA in combination demonstrates a significant impact on the regeneration of the main shoot [19]. In *Crataegus monogyna*, 6-BAP was one of the essential PGRs [20].

In our study, we investigated the effects of QL, MS and WPM media supplemented with constant concentration 0.25 mg/l 6-BAP and 0.5 mg/l GA3 on shoot regeneration (Fig. 3). Applied treatments were found to be suitable for target species with some adjustments.



A) QL *B)* MS *C)* WPM

Figure 3. Explants of Crataegus sanguinea germinated after 2 weeks of culture on different nutrient mediums supplemented with 6-BAP and GA3

The leaf lamina of the formed shoots was well developed on all nutrient media. However, based on visual observations, explants cultured in MS and WPM media exhibited signs of drying (Fig. 3). Thus, QL medium proved to be the most effective for the development of primary microshoots of *Crataegus sanguinea*.

This underscores the critical role of choosing the right nutrient medium for the main microshoot regeneration based on observed quantitative data (Table 3, Fig. 4). The highest regeneration rate was 76.6 %, with 23 out of 30 explants on QL medium. In contrast, the initial shoot formation rates were lower on both MS and WPM media, 66.6 % and 60 %, respectively.

Table 3

The effect of nutrient media on shoot regeneration

Treatments	Total number of cultivated explants, pcs.	Number of regenerated explants, pcs.	Percentage of regeneration, %
I — QL 0.25 mg/l 6-BAP and 0.5 mg/l GA3	30	23	76.6
II — MS 0.25 mg/l 6-BAP and 0.5 mg/l GA3	30	20	66.6
III — WPM 0.25 mg/l 6-BAP and 0.5 mg/l GA3	30	18	60.0

A study on Crataegus pinnatifida showed that successful regeneration of the main shoot occurred with 6-BAP in the range of 0.5 to 1.5 mg/l [21].

However, our experiment demonstrated that QL medium supplemented with 0.25 mg/l *6-BAP and 0.5* mg/l *GA3 yielded sufficient results for initial shoot formation of Crataegus sanguinea.*

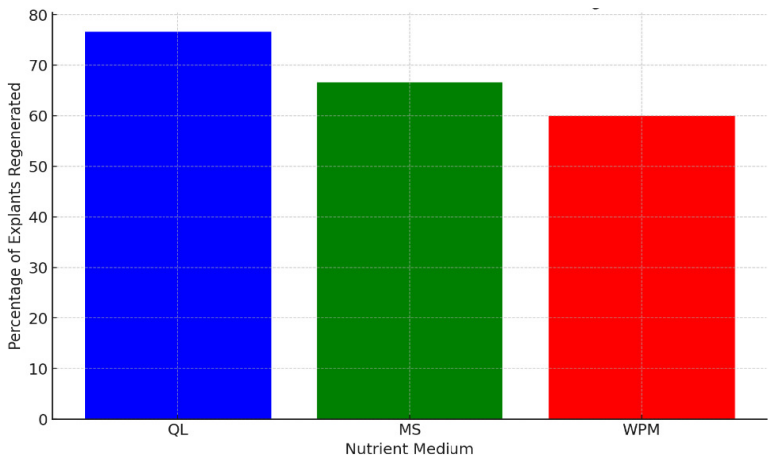


Figure 4. *Effect of nutrient media on shoot regeneration*

Shoot multiplication

In this study, we optimized a protocol for the *in vitro* multiplication of *Crataegus sanguinea* via the cultivation of regenerated explants in QL medium with 6-BAP, GA3 and IBA. We examined the effects of 6-BAP at different concentrations (from 0.25 to 0.75 mg/l) in combination with 0.5 mg/l GA3 and 0.01 mg/l IBA in QL medium. The results of the analysis of variability in morphometric characteristics in the observed *Crategus sanguinea* plants are presented in Table 4, which contains the summary data for all the studied treatments.

Table 4

The effect of 6-BAP concentrations on shoot proliferation of hawthorn (*Crataegus sanguinea)*

Treatments	PGR (mg/l)			35 Days after transplantation		
	6-BAP	GA3	IBA	Average number of shoots per explant, pcs.	Mean shoot height, cm	Number of leaves per explant, pcs.
I (Control)	-	-	-	3.26±0.11	1.50±0.09	13.07±0.24
II	0.25	0.5	0.01	2.34±0.08*	4.33±0.22*	10.33±0.17*
III	0.5			5.23±0.07*	6.77±0.18*	10.97±0.23*
IV	0.75			2.96±0.06	12.77±0.48*	11.87±0.35
Note – *The average difference is significant at the 0.05. Data are expressed as means ± standard error.						

It was found that the QL medium supplemented with 0.5 mg/l 6-BAP, 0.5 mg/l GA3, and 0.01 mg/l IBA resulted in high multiplication, with an average number of shoots per explant being 5.23 ± 0.07 (Table 4, Fig. 5). The plants appeared visually healthy, with an average shoot height and number of leaves per explant being 6.77 ± 0.18 cm and 10.97 ± 0.23, respectively.

The collected data suggest that the concentration of the cytokinin 6-BAP has a significant impact on the height, number, and quality of the developing plants of *Crataegus sanguinea*.

A) PGRs-free culture medium; B) 0.25 mg/l 6-BAP, GA3 0.5 mg/l, IBA 0.01 mg/l QL medium; C) QL 6-BAP 0.5 mg/l, GA3 0.5 mg/l, IBA 0.01 mg/l; D) QL 6-BAP 0.75 mg/l, GA3 0.5 mg/l, IBA 0.01 mg/l

Figure 5. Optimization of 6-BAP concentration for shoot proliferation of *Crataegus sanguinea*

Auxin and cytokinin are pivotal in regulating key stages of plant development in tissue culture. This is exemplified by research on *Allium cepa*, which has provided insights into the molecular mechanisms through which PGRs interact with *CDK* genes, influencing their activity and thereby regulating plant growth phases [22].

It was reported that the combination of cytokinins and auxins significantly influenced both the average number of shoots per explant and shoot length in sweet cherry (*Lapins Prunus avium L.*), where 6-BAP supplementation proved effective in promoting shoot multiplication [23]. This is consistent with our observations on *Crataegus sanguinea.* Additionally, it also was reported that the significant influence of 6-BAP supplementation on other *Crataegus* species [20]. The investigation on *Crataegus aronia* L. showed that media supplemented only with 6-BAP yielded the highest shoot production by promoting both shoot proliferation

and elongation. Higher shoot numbers were observed at 5.0 and 7.5 μM 6-BAP, while TDZ (Thidiazuron) caused significant callus formation [13]. Assessed shoot production among *Crataegus aronia, Crataegus pseudoheterophylla,* and *Crataegus meyeri* demonstrated variability in response to different combinations of PGRs. Notably, the combination of 1 mg/l NAA (Naphthaleneacetic acid) and 4 mg/l 6-BAP (6-Benzylaminopurine) was particularly effective, generating the highest average number of shoots after 6 weeks of cultivation [24].

Among the studied species, *Crataegus aronia* produced the highest percentage of successful explants, achieving 89.67 % success rate under this PGR combination. This indicates a strong propensity for shoot regeneration in *Crataegus aronia*, suggesting its potential for efficient shoot proliferation [24].

In contrast, *Crataegus meyeri* exhibited the longest average shoot length, reaching 22.67 mm. This was achieved under a different PGR concentration of 1 mg/l NAA and 1 mg/l 6-BAP, highlighting the species-specific response to growth regulators [24].

By considering above mentioned information we highlight the importance of optimizing PGR concentrations to each *Crataegus* species to maximize growth parameters.

Conclusion

The method employed in this experiment enabled effective shoot multiplication using auxiliary buds, bypassing the germination barrier and significantly reducing the propagation time. This approach facilitated the rapid and mass production of plant materials. Our findings insights are valuable for improving shoot proliferation protocols and enhancing the efficiency of *in vitro* cultivation of *Crataegus sanguinea*, which is valuable for its high fruit quality and ornamental, pharmaceutical, ecological, and rootstock importance. The use of QL media supplemented with certain concentrations of 6-BAP, GA3, and IBA can be recommended to enhance the multiplication of *Crataegus* spp. *In vitro* collection of *Crataegus sanguinea* has been created and will be used for further research.

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Өсімдік гормондарының *Crataegus sanguinea* *in vitro* жағдайындағы көбеюіне әсері

Зерттеу жұмысы өсімдік гормондарының *Crataegus sanguinea* микроөркендерінің көбеюіне қандай әсер ететінін зерттеуге бағытталған. Ол үшін бензиламинопуриннің келесі концентрациясы (6-БАП) 0,25, 0,5 және 0,75 мг/л, 0,5 мг/л гибберелл қышқылымен (ГҚ) және 0,01 мг/л индол-3-май қышқылымен (IBA) *Quoirin & Lepoivre* (QL) қоректік ортада зерттелді. Зерттеу нәтижелері бір эксплантқа ең көп өскіндер (5,23) ГК3 регенерациялық қоректік ортаға 0,5 мг/л, 0,5 мг/л 6-БАП, 0,5 мг/л GA3 және 0,01 мг/л IBA концентрациясында қосылу арқылы түзілетінін көрсетті. Алайда культивациялаудың 36-шы күнінде 0,75 мг/л 6-БАП, 0,5 мг/л GA3 және 0,01 мг/л IBA пайдалану арқылы эксплантағы өркеннің орташа ұзындығы (12,77 см) алынды. Атқарылған жұмыс негізінде *Crataegus sanguinea* өсімдігінің микроөскіндерін *in vitro* жағдайында көбейту үшін тиімді протокол ұсынылды. Бұл өз кезегінде микроөскіндердің саны мен ұзындығын жоғары деңгейде қамтамасыз етеді деп болжанады.

Кілт сөздер: қолтық бүршік, QL қоректік *ортасы*, өсімдік гармондары, микроөркендерді көбейту, *Crataegus sanguinea*.

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Влияние регуляторов роста на мультипликацию *Crataegus sanguinea* в культуре *in vitro*

В данном исследовании было изучено влияние регуляторов роста растений на пролиферацию дополнительных микропобегов у *Crataegus sanguinea*. Для этого были изучены следующие концентрации бензиламинопурина (6-БАП) 0,25, 0,5 и 0,75 мг/л, в комбинации с 0,5 мг/л гиббереллловой кислоты (ГК) и 0,01 мг/л индол-3-маслянной кислоты (IBA) на питательной среде *Quoirin & Lepoivre* (QL). Результаты исследований показали, что наибольшее количество побегов на эксплант (5,23) образовано добавлением в питательную среду регенерации ГК3 в концентрации 0,5 мг/л, 0,5 мг/л 6-БАП, 0,5 мг/л GA3 и 0,01 мг/л IBA. Однако средняя длина побегов на эксплант (12,77 см) была получена при использовании 0,75 мг/л 6-БАП, 0,5 мг/л GA3 и 0,01 мг/л IBA, на 36 день культивации. Настоящая статья представляет собой эффективный протокол для мультипликации микропобегов *Crataegus sanguinea*.

Ключевые слова: пазушные почки, питательная среда QL, регуляторы роста, мультипликация, *Crataegus sanguinea*.

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