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MICROCLONAL PROPAGATION OF THE VARIETIES OF HIGHBUSH BLUEBERRY *Vaccinium corymbosum* L.

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The aim of the work was to determine optimal conditions for clonal reproduction, growth and development of different varieties of *Vaccinium corymbosum* from the foreign selection. The objects of research were 5 varieties of highbush blueberry: Bluejay, of early ripening period; Bluecrop, Bluegold, Legacy, of average ripening; Aurora, of late ripening. Optimal conditions of explant surface sterilization have been selected depending on their type and effectiveness of used disinfectants. Maximum quantity of viable sterile regenerates was obtained using the mixture of sterilizing solutions of alcohol and bleach "Belizna" with Tween. Effectiveness of microclonal reproduction depending on the composition of nutrient agar medium with organic compounds on macro- and microsalt basis WPM (Woody Plant Medium) with addition of the growth-regulator of cytokinine action 6-γ, γ-Dimethylallylaminopurine (2iP) was evaluated according to regenerative activity of explants and shoots reproduction coefficient. The results of experiments showed that effectiveness of phytohormones action and their concentrations depended on genotype properties of every variety of *V. corymbosum*. It has been established that the growth activity and shoots multiplications changed depending on the concentration in nutrient medium 2iP. Maximum values of reproduction coefficient for Bluecrop and Bluegold varieties and shoots height were determined on the medium with 4 mg/l 2iP. For Aurora and Legacy the regenerative ability was the highest with concentration 8 mg/l 2iP and for Bluejay, 10 mg/l 2iP.

Key words: *Vaccinium corymbosum*, clonal micropropagation.

Cellular technology in crop production based on the propagation *in vitro* of organs, tissues, cells, and isolated protoplasts of higher plants are used to create genetically diverse plant material, particularly for obtaining new varieties of plants, accelerated vegetative propagation of forms with improved economic characteristics and increased productivity [1, 2].

High bush Blueberries (*Vaccinium corymbosum* L.) — the most common species of the genus; varieties are quite frost-resistant and are cultivated almost throughout the whole territory of the United States as well as Canada, Australia, New Zealand, China and European countries [3–5]. Poland, in particular, has become one of the world leaders in the cultivation of berries over the past decade [6]. Microclonal reproduction of blueberries and other economically valuable and rare species of plants has been widely implemented into practice in Belarus [7, 8]. In Ukraine it is a new perspective culture for the fruit production. It is rare in nature and culture compared to traditional raspberries, strawberries, black currants, but it is much more attractive for industrial and individual gardening due to its taste and medicinal properties.

In order to increase production volumes and quality of recovered seedlings of valuable blueberry varieties new high technologies are used, including microclonal reproduction that takes key place as an alternative to traditional technologies of vegetative propagation. The small size of explants used for micropropagation, their surface sterilization, sterile aseptic cultivation on nutrient growth medium heals received blueberry plants from

possible infection by nematodes and bacterial pathogens, and allows largely rid of viruses, viroids and mycoplasmas [8].

The literature on methods of microclonal reproduction of *V. corymbosum in vitro* is highly contradictory regarding the type of culture medium and its hormonal composition [9–12]. Since many commercial varieties of blueberries have low regenerative capacity, intensity of ontogenetic processes in cell cultures are increased due to the selection of specific culture conditions and nutritient medium components, namely phytohormones, growth regulators and synthetic substances as effective non-hormonal origin stimulants of morphogenesis. Since cytokinins and auxins significantly increase the cost of microclonal reproduction technology to produce a sufficient amount of healed planting material of different varieties of blueberries, selection of the composition of the nutrition medium is required. It will significantly minimize the cost of cloning and balance the regeneration capacity of explants, the term during which regenerants are obtained and the quality of shoots.

Microclonal reproduction of high bush blueberry is not practiced a lot in Ukraine, and the area of industrial plantations in spring 2014 did not exceed 600 hectares. Blueberries are one of the most expensive consumer berries, which significantly increased global market demand over the past decade. Selection of the conditions for microclonal propagation of different varieties of blueberries will help speed up their implementation into industrial production and contribute to increasing production areas of culture, its plantations will become a source of additional income for small farmers, or basis for the functioning of large farms.

The aim of the work was to determine optimal conditions for aseptic microclonal propagation and development of guidelines on growing high bush blueberry culture that will make it possible to get healed plant material, increase output and improve the quality of planting material of varieties.

Materials and Methods

The material for the research were fragments of young shoots with buds of 5 varieties of high bush blueberry *V. corymbosum* of foreign selection, early ripening variety — Bluejay; average ripening — Bluecrop, Bluegold, Legacy , late ripening — Aurora.

Aseptic culture of high bush blueberry *in vitro* was obtained by method that has been modified by us [13]. The selection of explants was carried out using fragments of the shoots with two or three axillary lateral buds 2–3-year-old from donor plants of different varieties of blueberries. Sterilization of plant material was performed using this protocol: tap water + 0.01% Tween (30 min), 70% ethanol (30 s), 10% — 15% — 20% solutions of sodium hypochlorite (“Bleach”) + 0.001% Tween (10 min, 15 min, 20 min) and triple washing in sterile distilled water for 20 min; tap water + 0.01% Tween (30 min), 70% ethanol (30 s), 0.1% or 0.01% solutions of mercuric chloride (1 min) and triple washing in sterile distilled water for 20 min. Surface-active agent Tween 20 (Phyto Technology Laboratories, USA) was used for better penetration of disinfection substances into the epidermal tissue of the shoots.

For microclonal high bush blueberry propagation nutrient agar medium with 2.41 g/l Woody Plant Medium (WPM) (Phyto Technology Laboratories, USA) was used. It was modified depending on the variety of growth regulators 4 mg/l, 6 mg/l, 8 mg/l and 10 mg/l 2iP — 6-(γ, γ-dymetylalilamino) purine (Phyto Technology Laboratories, USA); 40 mg/l and 80 mg/l adeninehemisulphate (Phyto Technology Laboratories, USA); 0.5 mg/l, 1 mg/l zeatin (Sigma); 50 mg/l and 100 mg/l chelated iron Sequesrene 138 (Phyto Technology Laboratories, USA); vitamins: B_1 (0.05%), B_6 (0.05%), PP (0.1%), C (50 mg/l) (Ukraine), 30 g/l sucrose (Ukraine); control — modified growth medium WPM, without growth regulator 2iP. Cultivation of sterile explants was carried in 450 ml glass jars (15 pieces per capacity) on the medium with 0.5% agar (Phyto Technology Laboratories, USA), pH 5.

Shoots were cultured in controlled conditions: light intensity from fluorescent lamps OSRAM L36W / 77 Fluora — 3000 Lux, temperature — 24 ± 2 °C, relative humidity — 70% and 16-hour photoperiod. To compare the efficiency of microclonal reproduction of high bush blueberries depending on the composition of the nutritient medium and micro-climatic conditions for the cultivation, explants’ regenerative ability was evaluated, the features of the shoots, variability of growth options regenerants and their reproduction coefficient — the ratio of shoots that developed from a bud (RC) were analyzed on a 6 week. Average RC was determined by mathematical statistics [14]. Regenerants’ height was measured on a sheet of graph paper.

All experiments were performed in 3-fold repetition. For the studied varieties in each version of the experiment, at least 150 regenerants (10 cans and 15 explants) were analyzed. The research results were processed statistically.

Results and Discussion

The ability to regenerate under *in vitro* conditions largely depends on the efficiency disinfectors used. For sterilization using affordable and relatively inexpensive disinfective substances, including the 70% ethanol, mercuric chloride solutions and "Bleach" with Tween 20.

Under the action of mercuric chloride 0.01% and 10% — and 15% solutions of "Bleach", almost 70–75% contamination of plant material was observed (Table 1).

In cases of using mercuric chloride, results showed damage (browning) of plant tissues and the presence of fungal and bacterial infections when explants were sterilized in 70% ethanol (1 min) and 0.01% of mercuric chloride (1 min). The first signs of contamination of *V. corymbosum* explants after exposure to 70% ethanol (1 min) and solutions with low concentrations of "Bleach" + Twin 20 (10 min) were exhibited at the 5th day of cultivation — 75% of the plants were infected.

As a result of the selection of experimental sterilization conditions, these conditions were defined as optimal: 70% ethanol (1 min) and 20% solution of "Bleach" + 0.001% Tween 20 (20 min). This resulted in receiving up to 90–100% of viable explants. Lack of bacterial and fungal infection on the 14–15 th day and later gave reason to consider blueberry garden culture aseptic. Such conditions of explants sterilization were used further. According to the literature, NaOCl are less toxic to explants of plant tissue compared to mercury compounds, and quite effective in the removal of plant pathogens [15].

The cultivation of *V. corymbosum* explants was performed on WPM nutritient medium [16], as most authors confirm that its mineral composition is best balanced for reproduction and growth *in vitro* for many plants, including blueberry garden [17–20]. However, the author's data on the hormonal composition of the culture medium are widely divergent and often contradictory [21–23]. We investigated the influence of hormonal and mineral nutrient composition of the medium to enter explants of high bush blueberry into the culture *in vitro*. The influence of different concentrations of 2iP, mineral compounds and vitamins on the height and number of shoots and number of full regenerants for microclonal propagation of varieties of high bush blueberries were analyzed. Regenerative ability of explants was assessed by the variability of growth parameters and coefficients of reproduction of regenerants.

Growing conditions — lighting, temperature, humidity, [24] as well as 2iP concentration in the culture medium greatly affect the height of the shoots of varieties of *V. corymbosum in vitro* (Table 2).

For Bluecrop and Bluegold varieties, the maximum height of shoots was determined on medium with 4 mg/l 2iP. Larger (6 mg/l) concentration of growth regulator inhibits growth of regenerants by 35–40%. For

Table 1. **Effect of sterilizing solutions on viability of *Vaccinium corymbosum* explants**

Exposition to sterilizing solutions, min	Aseptic explants,%	Viable explants, %	Coloration of explants
70% ethanol (1 min) + 0.01% mercuric chloride (1 min)	30	10	Green-and-brown
70% ethanol (1 min) + 0,1% mercuric chloride (2 min)	90	35	Brown
70% ethanol (1 min) + 10% "Bleach" + 0.001% Tween 20 (10 min)	25	15	Green
70% ethanol (1 min) + 15% "Bleach" + 0.001% Tween 20 (15 min)	80	75	Light green
70% ethanol (1 min) + 20% "Bleach" + 0.001% Tween 20 (20 min)	97	90	Light green

Bluecrop and Bluegold varieties higher concentrations of the regulator are toxic: under the influence of 8–10 mg/l 2iP the termination of shoot growth, and the long cultivation — browning of explants was noted. For Aurora and Legacy varieties, 2iP concentration of 8 mg / l positively influenced the height of shoots and their rate of growth, while Bluejay had the most effective growth under regulator concentration of 10 mg/l (Fig. 1).

Reproduction coefficient (RC) is a measure of efficiency of the microclonal propagation of shoots *in vitro*, which determines the number of newly formed shoots. It was established that the value of the RC of analyzed varieties largely varied depending on the concentration of 2iP in the nutritient medium. With increasing of the concentration of 2iP 4 to 8mg/l, RC coefficient of the nutritient medium WPM were increased for Legacy, Aurora and Bluejay varieties. For Aurora and Legacy varieties, regenerative

Table 2. **Height of regenerants of *Vaccinium corymbosum* varieties depending on 2iP concentration in nutrient medium**

Concentration of 2iP, mg/l	Height of regenerants of *Vaccinium corymbosum* L. varieties, cm				
	Aurora	Bluecrop	Bluegold	Bluejay	Legacy
Control	0.27 ± 0.017	0.38 ± 0.021	0.63 ± 0.034	0.36 ± 0.009	0.91 ± 0.036
4	1.22 ± 0.46*	3.03 ± 0.98*	3.46 ± 1.05*	–*	3.39 ± 0.87
6	2.56 ± 0.87*	2.01 ± 0.68*	2.11 ± 0.67*	0.84 ± 0.35	2.62 ± 1.02
8	3.58 ± 1.29*	–	–	1.25 ± 0.53	4.11 ± 1.04
10	2.92 ± 1.01*	–	–	2.59 ± 1.02	3.54 ± 0.93

Note: hereinafter * $P < 0.05$; –* no regenerants formed.

Fig. 1. **Measuring the height of *Vaccinium corymbosum* shoots of the Bluejay variety on nutrient medium WPM with 10 mg/l 2iP**

ability was at a maximum concentration of 8 mg/l 2iP in the nutritient medium, and for Bluejay — 10 mg/l. The results of the RC analysis *in vitro* of *V. corymbosum* varieties are presented in Table 3. Based on the found results, it has been established that for multiplication and development of shoots of Bluegold and Bluecrop, inverse dependence on the dose of growth stimulator is typical: maximum number of RC was recorded at the lowest concentration of 2iP in the nutritient medium — 4 mg/l, with increase of the dose of regulator to 6 mg/l, there is a marked decrease in its RC value; the concentrations of 8 mg/l and 10 mg/l were inhibitory for investigated varieties.

Adding 0.4 mg/l indolilacetic acid (IAA) to the medium resulted in emergence of large number of new shoots. Specifically for regenerants of Bluegold varieties, RC value increased to 2.3 (Fig. 2), but the growth of shoots was slowed down and the shoots browned overtime. Under the influence of gibberellic acid (GC) in the same concentration, elongation of shoots intensified, but their number did not increase, the RC coefficient was 1 in this variety. Zeatin at a concentration of 0.5 mg/l slightly stimulated the development of buds, and increase the concentration of the hormone to 1 mg/l caused intense callusogenesis that inhibited growth of regenerants.

Table 3. **Effect of different 2iP concentrations on reproduction coefficient of *Vaccinium corymbosum* varieties *in vitro***

Concentration of 2iP, mg/l	Reproduction coefficient of Vaccinium corymbosum varieties				
	Aurora	Bluecrop	Bluegold	Bluejay	Legacy
Control	0.44 ± 0.073	0.61 ± 0.028	1.12 ± 0.023	0.26 ± 0.11	2.25 ± 0.17
4	2.29 ± 0.93*	2.21 ± 0.78*	4.38 ± 1.15*	–*	3.8 ± 0.85
6	3.89 ± 1.29*	1.21 ± 0.29*	2.05 ± 0.41*	1.03 ± 0.50	3.2 ± 1.11
8	6.01 ± 1.94*	–	–	1.42 ± 0.68	5.34 ± 1.96
10	4.33 ± 1.47*	–	–	2.4 ± 1.07	5.04 ± 1.12

Fig. 2. **Culture of *Vaccinium corymbosum* variety Bluegold on WPM with 4 mg/l 2iP concentration**

To initiate the growth of buds of Aurora variety under 0,4 and 0,8 mg / l concentration of IAA in the culture medium were ineffective, acceleration of buds' growth were not obtained. Only increasing the concentration of IAA to 4 mg/l stimulated the growth of shoots, but the average value of RC did not exceed 1. Long shoots cultivation lead to the destruction of chlorophyll in leaves. Transplantation of etiolized shoots onto the nutritient medium with a higher concentration of 2iP (6 mg/l or 8 mg/l) contributed to the restoration of chlorophyll and more active growth. 1 mg/l of zeatin initiated intensive callus growth, while lower concentration (0.5 mg/l) of hormone accelerated buds expansion and the shoots formation, but their further growth, as well as callusogenesis, was not observed.

Reproduction of regenerants of Bluecrop variety was complex and it was difficult to identify the components of the nutrient medium [25, 26]. The use of 0.4 mg/l and 4 mg/l IAA was ineffective for buds developing [27]. The influence of 0.8 mg/l IAA improved their growth, but usually one leaf grew actively, meanwhile shoots did not become longer. The use of 0.8 mg/l of gibberillic acid (GC) stimulated phase of shoot tension, but had no effect on the rate of their development — they grew slowly and eventually browned. Adding 0.5 mg/l of zeatin stimulated callusogenesis as well as the appearance of shoots, but their growth was too slow. Perhaps higher concentrations (2–5 mg/l) of zeatin, as it is indicated by many authors [28–30], would increase the number of microshoots and accelerate their morphogenesis, but the effect of hormone in such concentrations, was not investigated by us because it is too expensive and uneconomical.

Thus, the results of the research show that the effectiveness of the various phytohormones and their concentrations are determined by the characteristics of each genotype of *V. corymbosum* varieties. Summary of experimental data gives reason to confirm that for obtaining regenerants with a moderate amount of shoots of defining quality in the culture medium WPM, the concentration of growth regulator of cytocinines action 2iP is crucial. The optimal conditions of *in vitro* propagation for the 5 varieties of high bush blueberry *V. corymbosum* were selected.

The study was conducted in the Department of Plant ecomorphogenesis of the Institute of Ecology of the Carpathians NAS of Ukraine.

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МІКРОКЛОНАЛЬНЕ РОЗМНОЖЕННЯ СОРТІВ ЛОХИНИ ВИСОКОРОСЛОЇ *Vaccinium corymbosum* L.

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Метою роботи було визначити оптимальні умови для клонального розмножения, росту і розвитку різних сортів *V. corymbosum* закордонної селекції. Об'єктом дослідження слугували регенеранти 5 сортів лохини високорослої: раннього терміну дозрівання — Блуджей (Bluejay); середньостиглі — Блукроп (Bluecrop), Блуголд (Bluegold), Легасі (Legacy); пізньостиглий — Аврора (Aurora). Підібрано оптимальні умови поверхневої стерилізації експлантів залежно від їхнього типу та ефективності використаних дезінфікаторів. Максимальну кількість життєздатних стерильних регенерантів отримали, використовуючи суміш стерилізувальних розчинів спирту і «Білизни» з Твіном. Ефективність мікроклонального розмноження залежно від складу живильного агаризованого середовища з органічними сполуками на макро- й мікросольовій основі WPM (Woody Plant Medium) із додаванням регулятора росту цитокінінової дії 6-(γ, γ-диметиламіламіно) пурину (2iP) оцінювали за регенераційною активністю експлантів та коефіцієнтом розмноження пагонів. Результати проведених досліджень свідчать, що ефективність дії фітогормонів та їхніх концентрацій залежала від особливостей генотипу кожного сорту *V. corymbosum*. Встановлено, що активність росту і мультиплікації пагонів змінювалися залежно від концентрації в живильному середовищі 2iP. Для сортів Bluecrop і Bluegold максимальні значення коефіцієнта розмноження і висоти пагонів визначено на середовищі WPM з 4 мг/л 2iP. Для Aurora і Legacy регенераційна здатність була найбільшою за концентрації 8 мг/л 2iP, а для Bluejay — 10 мг/л 2iP.

Ключові слова: *Vaccinium corymbosum*, мікроклональне розмноження.

МИКРОКЛОНАЛЬНОЕ РАЗМНОЖЕНИЕ СОРТОВ ГОЛУБИКИ ВЫСОКОРОСЛОЙ *Vaccinium corymbosum* L.

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Целью работы было определение оптимальных условий для клонального размножения, роста и развития различных сортов *V. corymbosum* зарубежной селекции. Объектом исследования служили регенеранты 5 сортов голубики высокорослой: раннего срока созревания — Блуджей (Bluejay); среднеспелые — Блукроп (Bluecrop), Блуголд (Bluegold), Легаси (Legacy); позднеспелый — Аврора (Aurora). Подобраны оптимальные условия поверхностной стерилизации эксплантов в зависимости от их типа и эффективности использованных дезинфикаторов. Максимальное количество жизнеспособных стерильных регенерантов достигнуто при использовании смеси стерилизующих растворов спирта и «Белизны» с Твином. Эффективность микроклонального размножения в зависимости от состава питательной агаризованной среды с органическими соединениями на макро- и микросолевой основе WPM (Woody Plant Medium) с добавлением регулятора роста цитокининового действия 6-(γ, γ-диметилалиламино) пурина (2iP) оценивали по регенерационной активности эксплантов и коэффициенту размножения побегов. Результаты проведенных исследований свидетельствуют о том, что эффективность действия фитогормонов и их концентраций зависели от особенностей генотипа каждого сорта *V. corymbosum*. Установлено, что активность роста и мультипликации побегов изменялись в зависимости от концентраций в питательной среде 2iP. Для сортов Bluecrop и Bluegold максимальные значения коэффициента размножения и высоты побегов определены на среде WPM с 4 мг/л 2iP. Наибольшая регенерационная способность для Aurora и Legacy была при концентрации 8 мг/л 2iP, а для Bluejay — 10 мг/л 2iP.

Ключевые слова: *Vaccinium corymbosum*, микроклональное размножение.