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Plant Growing

Production of Sapling Material of Blueberry in *In Vitro* Culture

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ABSTRACT: The in vitro culture method widely used in the process of working on selection and genetics of plants for receiving the medical, pharmaceutical and other forms of agricultural, rare, endangered plants is considered in the paper. Blueberry (Vaccinium uliginosum) belongs to the that kind of plants. Blueberry is not sufficiently studied in vitro that is related to the morphogenetic peculiarities of the plant in vitro. For the selectionists it is of great practical and theoretical significance to solve the mentioned problem. The issues of microclonal propagation of blueberry in vitro, production of the regenerant plants, their rooting and acclimatization are studied in the present work. *© 2018 Bull. Georg. Natl. Acad. Sci.*

Key words: generative, explant, juvenile, clonal, micro-propagation, rejuvenated

The plant tissue culture studies the development of the plant cells, tissues and organs on an artificial nutrient medium in vitro. The cell, tissue and organ culture of plants, i.e. cellular technology is a fast-growing direction, which developed from laboratory methods into a real field of science and is justly called the "growing cell" biology.

In 1949, Limasset and Cornuet found that the meristem cells of a plant are often virus-free. In 1952, Morel and Martin used that fact and decided to obtain an in vitro culture from the meristem cells.

The history of blueberry began at the end of the 19th century, when the wild forms of blueberry were intensively cultivated in the US. In the 80s of the 20th century, there were registered 45 varieties of rapidly growing blueberry. Famous scientists, farmers and gardeners of 13 American States were all involved in the work. Almost in the same period, selection works were intensively going on in Canada. Today, blueberry grows in the USA, Canada, Europe, New Zealand, Australia, Japan and Ukraine.

In 2006, the blueberry plants were first brought to Georgia from the United States. The experimental saples were planted in the village of v. Simoneti, Imereti region, where the first harvest was obtained in 2009. Today, about 150 varieties of blueberry are cultivated including: Bluecrop, Chandler, Legacy, Berkeley, Patriot, Brigitte, Duke, Misty, Sunrise, Spartan, Toro, O'neal,

Elizabeth, Blugold blueberry etc. Of them over 9 varieties are introduced to Georgia.

The USA is the leader in the world in blueberry production, where in 2009 about 166 786 tonnes of blueberry was manufactured. Along with the USA, the other dominant countries in blueberry raising and production are Australia and Canada. South Korea and China are also among the leading countries. Blueberry became an important culture in Turkey and Europe.

Material and Methods

The object of study was blueberry of heather family (*Ericaceae*). It is a low, 15-40 cm tall deciduous bush with green ribbed sprouts and horizontally spread roots in soil. Their alternate bright green leaves on the 15-25 mm long branches are short-stalked and glossy; they are egg-shaped or ovate with fine and jagged edges.

In the modern taxonomy the blueberry genus (*Vaccinium*) is represented by about 200 species. There are four species of the mentioned genus spread in Georgia. Three of them - mountain blueberry (*Vaccinium Myrtillus*), bog blueberry (*Vaccinium uliginosum*) and lingonberry (*Vaccinium Vitis-ideaea*) belong to boreal culture and are spread in alpine and subalpine zones [1].

The Caucasian or the tall whortleberry (*Vaccinium Arctostaphylos*) is mainly found in the Colchic forests of the middle and lower mountain zones of the north-eastern Antalya. It is widespread in almost every region of Georgia: Adjara-Guria mountains, Abkhazia, Racha-Lechkhumi, Imereti, Kartli, Svaneti, Tush-Pshav-Khevsureti. The fruit ripens in July-August.

The wild form of blueberry grows well in all kinds of arid, rocky and sandy soils with maximum harvest on acidic soils. It does not require much light or moisture and is characterized by superficial root system and frost resistance (– 20 -25°C).

The blueberry fruit contains: sugar (pentose, fructose), catechins, pectins, tannin substances; ascorbic, apple, lemon and milk acids; flavonoids; B, C, V vitamins and other useful substances.

Today, out of the cultivated varieties the blueberry is most common in the world. Its saplings are propagated by cell culture method *in vitro* (in a tube or flask) in laboratory conditions. Blueberry requires rather specific conditions. For normal growth and development and for great productivity of the plant soil acidity (ph) should be 4.0 - 5.0 and the plot should be well-drained, friable, sunlit and wet. The blueberry is quite successfully cultivated in the Black Sea regions of Western Georgia on the plots of the former tea plantations.

Fig. 1. Cultivated blueberry.

The blueberry fruit is sweet with slightly sour taste. It is used for production of jams and compotes as well as for medicinal purposes. It regulates the gastrointestinal tract. Besides, it is the best remedy for rheumatism and various inflammations, for recovery of the eyesight. The tea made from the blueberry fruit and leaves is used for treatment and prophylaxis of diabetes, urethral stone pathology, eye inflammations and other diseases [2].

In modern experimental biology, the cell, tissue and organ culture in vitro method is successfully implemented aiming to improve the existing varieties of agricultural species and create the new ones. This method is widely used for propagation of the poorly reproducible relic, endemic or rare forms [3].

Tissue culture plays important role in producing genetically identical plants. To that end, one of the methods of cellular technology called microclonic propagation is used. In the experiment the apical

Table 1. Sterilizing substances

Sterilizing solution	Concentration, %	Exposition min	Uninfected, %	Viability
Chloramine B	15	20	20	82
	15	20	32.0	78
•Commercial chlorine	25	20	35.0	100
solution	50	20	49.0	100
Diocide	0.1	15	34.	93.0
	0.2	15	58.0	92.0
	0,5	15	96.0	69.0

parts of the sprout, the leaves, the buds with axillar meristematic tissue capable to turn into growing sprouts are used as explants. By means of the mentioned method both grassy and timber plants can be rapidly propagated. It should be noted that in the open ground the plants are inhabited by a lot of microflora of different etiology. In most cases they do not have any influence on the plant viability, rather many of them are symbiotic with the host plant. Cultivation of plants in *in vitro* culture requires cleaning from microflora. The sterilizing agents are of chemical character causing toxic action of different intensity against the living tissues, therefore, they are selected empirically. The anatomic-morphological structure of the explant is very important for estimation of the intensity of the sterilizing agents.

The main objectives of the *in vitro* method is: to develop special conditions for introduction of the plant into the *in vitro* culture; to select and optimize the processes of microclonic propagation; to study the influence of physical conditions of growth regulators and cultivation on each stage of microclonic propagation and to study the process of acclimatization of the rooted regenerant plants.

In the first step of the experiment one of the challenging and hard-working processes is to create optimal conditions for sterilization and correct selection of the sterilizing agents. The following solutions are used as sterilizing substances:

Commercial chlorine solution (sodium hypochloride - 50/50 and 25/75 ratio);

Diocide (ethinole-mercuric-chloride – 0.1/0.2 and 0.5% water solutions of cetyl-pyridinium chloride in the ratio of 1/2);

10 -15% water solution of chloramine B, which is added by a few drops of Tween-80 as the superficial sterilization activating agent [4-6].

The steps of micropropagation:

First step: To obtain an initial explant; to introduce the explant into in vitro system; to create clean, well-growing virus-free material;

Second step: Micropropagation and maximum increase of the amount of microlones at the expense of new buds;

Third step: Rooting of the propagated sprouts; normal development of root system is necessary; after that the plant is ready to be transferred into soil.

Some researchers also identify the fourth step, in particular, preparation of the plant for transferring into soil [1].

Research

The goal of the research was introduction of the blueberry into *in vitro* culture and its microclonic propagation. To that end, appropriate conditions were selected for introduction of blueberry into in vitro culture and for its microclonic propagation. The influence of physical conditions of the growth regulators and cultivating was studied during microclonic propagation, and the process of acclimatization of the rooted regenerant plants was studied in greenhouse conditions.

Table 2. Production of blueberry *de novo* sprouts on different nutrient mediums

Name of the research object		Murashige-Skoog nutritient medium(modified)		Anderson nutrient medium		
	Amount of explant	Able to produce *de novo* sprouts, amount	%	Amount of explant	Able to produce *de novo* sprouts, amount	%
Blueberry	150	127	85	150	58	39

The following sterilizing substances were studied and tested:

•Commercial chlorine solution. Composition: sodium hydrochloride - water in the ratio of 50/50 and 25/75;

•Diocide. Composition: 0.1/0.2 and 0.5% aqueous solution; ethinole-mercuric-chloride and cetyl-pyridinium chloride in ratio;

•10-15% aqueous solution of chloramine B, which was added by a few drops of "Tween-80" as a superficial sterilization agent.

As Table 1 shows, the outcome of the virus free material was 20 and 32%, respectively. Comparatively better results showed commercial chlorine solution: the amount of virus free material was 35-49%. The positive effect of superficial sterilization was achieved by using the preparation Diocide. The low level of infection was provided by 0.5%- solution, under the action of which the amount of virus free material achieved 86%. However, the viability of aseptic cultures was lower compared to all the other tested sterilizing agents. Due to strong toxicity of Diocide its high concentration caused intoxication of the tissue. The results of the surface sterilization procedure showed that the concentration of the sterilizing substance, the delay of the explant in the solution and the type of the explant had an influence on the yield of viable aseptic material.

Initial material of the experiment was taken from the blueberry mother plant in both closed and open ground conditions. The following surface plants were used:

•The axillary and apical buds of plants, the leaves and stems of young sprouts of the plants growing in nurseries;

The axillary and apical buds of plants vegetated in greenhouses.

The following plants of the juvenile phase were used:

•The sleeping and apical buds, stem tissue and leaves of the greenhouse plants of 3–4 months;

•The whole sprout without root obtained from the crops of in vitro cultivated seeds;

•The in vitro cultivated sprouts, buds and leaves of the fully-grown plant.

Two nutrition mediums - the modified Murashige-Skoog and Anderson's nutrition mediums were used in the experiment. The axillary and apical buds of blueberry were placed on the nutrition mediums of above-said mineral composition.

Murashige-Skoog nutrition medium contained: Vitamin C (1.0 mg/l), vitamin B1 (1.0 mg/l), nicotine acid (0.5 mg/l), inositol (1.0 mg/l), double content of iron chelate; sucrose (20 m/l), agar-agar (8 g/l).

Anderson's medium contained: macrolements - NH_4NO_3 (400 mg/l); KNO_3 (480 mg/l); $MgHO_4$. $7H_2O$ (180 mg/l); Na_2PO_4 . H_2O (330.6 mg/l); CaCl (322.2 mg/l); NH_4NO_3 (mg/l); microelements - M_3BO_3 (6.2 mg/l); $MnSO_4$. $4H_2O$ (16.9 mg/l); $ZnSO_4$. $7H_2O$ (8.6 mg/l); KJ (6.3 mg/l); Na_2MnO_4. $2H_2O$ (0.25 mg/l); $CuSO_4$. $5H_2O$ (0.0025 mg/l); $CoCl_2$. $5H_2O$ (0.025 mg/l); Ferrous sulphate - $FeSO_4.7H_2o$ (55.7 mg/l); Na_2 (74.5 mg/l); Vitamins - B1, B6, PP (0.5 mg/l), C (1.5 mg/l); mezo-inosite (100 mg/l); phytohormones (0.5 mg/l); Sugar - sucrose (20 g/l).

Out of all plant-regenerators (150) 23 plants died during acclimatization, and 127 plants were found able to produce de novo sprouts on the

Murashige-Skoog (modified) nutrient medium. On the Anderson's nutrient medium out of 150 plant-regenerators 92 died during acclimatization and 58 were found able to produce de novo sprouts (Table 2).

Based on the above said, the Murashige-Skoog (modified) nutrient medium (Fig. 2) is more productive for blueberries than Anderson's medium.

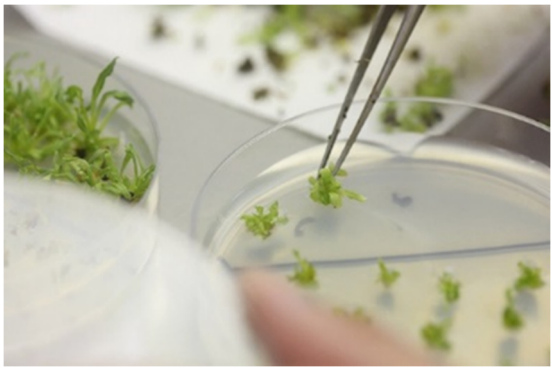

Fig. 2. Murashige-Skoog nutrient medium.

According to the results of the experiment, the explant cultivation on different nutrient mediums revealed different effects. The buds developed on the Andersson's nutrient medium containing mineral salts were weak with a mean height of no more than 2 - 5 mm. Development of new leaves was very slow and weak. At the end of the passage the leaves faded and fell into the flask leaving the main stem of the sprout bare.

On both nutrient mediums there were found morphogenic calluses developed in the basal part of the explant, where some purple morphogenetic nodes appeared that formed the primordial buds. However, those buds were not characterized by the morphogenesis of the stem. Therefore, Anderson's nutrient medium was disregarded. Germination of *de novo* buds, emergence of advent buds and induction of the axillary buds proceeded successfully on the Murashige-Skoog nutrient medium, where normal viable sprouts developed at the end of the passage.

Thus, right selection of the nutrient medium is an important step in the process of micro-propagation, which is followed by the third step of rooting and acclimatization of regenerant plants.

In the phytotron, where the day-night regime is kept, i.e. it is dark for 8 hours and light for 16 hours and the temperature is 22-24°C, the plant sprouts (cleaned of the morphogenic callus formed in the basal part) were placed on the nutrient medium added by the root stimulator "Kornevin" (Fig. 3). They were delayed there for about a month and a half until the plant height reached 1.5 cm, then they were transferred into the sterile soil (sand and peat, 50:50) in the cultivating vessel and were covered by a polyethylene bag. They stayed covered during a month but were uncovered once a day for acclimatization. After that they were uncovered and placed in the greenhouse, where the temperature regime varied within 20-25°C.

Fig. 3. Removing morphogenic calluses in the basal part.

Two months later, the regenerant plants were transferred into pots in greenhouse conditions, and after 10-15 days in open ground.

In the period of acclimatization, the regenerant plants retained dark green color, straight stem and the morphology of the leaves. And the length and thickness of the root system grew along with putting out of branches.

Finally, in the last step of development the plant grew and developed rapidly. Over the following two weeks the plant growth in height was about 3-4 times more intensive compared to the initial growth. In a month after transferring the plant into soil the height of the plant above the ground was over 40-50 cm and the roots system gradually took the form of a brush-like root system. Out of the total

number of regenerant plants (127 plants) 23 died during the acclimatization period, and 104 plants, i.e. 82% acclimatized.

Conclusion

Thus, according to the results of the research, it can be concluded that it is an optimal condition to use a 0.5% diocide solution to obtain a virus free, viable explant for superficial sterilization of the blueberry initial explant. The Murashige-Skoog (modified) nutrient medium should be chosen to receive the mass sprouts of blueberry in *in vitro* culture. Blueberry plant material can be obtained by the method of in vitro culture.

მცენარეთა ფიზიოლოგია

ლურჯი მოცვის სანერგე მასალის მიღება **in vitro** კულტურაში

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განხილულია in vitro კულტურის მეთოდი, რომელიც ფართოდ გამოიყენება მცენარეთა სელექციურ-გენეტიკური სამუშაოების შესრულებისას, კერძოდ, სასოფლო-სამეურნეო, იშვიათი, გადაშენების გზაზე მყოფი სამკურნალო ან სამკურნალწამლო და სხვა ფორმების მისაღებად. ასეთ მცენარეთა რიცხვს მიეკუთვნება ლურჯი მოცვი (Vaccinium uliginosum). In vitro კულტურაში ლურჯი მოცვი პრაქტიკულად ნაკლებადაა შესწავლილი, რაც in vitro-ში მცენარის მორფოგენეზის თავისებურებებთანაა დაკავშირებული. სელექციონერებისათვის აღნიშნული პრობლემის გადაჭრას პრაქტიკული და თეორიული მნიშვნელობა აქვს. წარმოდგენილი კვლევა ეხება ლურჯი მოცვის in vitro სისტემაში მიკროკლონურ გამრავლებას, მცენარე-რეგენერანტების მიღებას, მის დაფესვიანებას და აკლიმატიზაციას.

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