Biotechnology methods in study of Vaccinium uliginosum L. and Vaccinium myrtillus L. in Armenia

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Abstract. The in vitro methods of Biotechnology have been used for analyzing of germination stages and grow conditions of Vaccinium L. subgenera spread in Armenia (Vaccinium myrtillus L. and Vaccinium uliginosum L.). These studies reveal that optimum conditions for in vitro germination of wild blueberry Vaccinium myrtillus and Vaccinium uliginosum seeds were: cold stratification 4-5 °C for 8 weeks, wherein the percentage of germinated seeds was 74.2 – 88.3%. The regenerative potential of shoot and root formation of the analyzed subgenera’s depends on the grow regulators composition of the medium. This Vaccinium L. in vitro study can be used for future using in breeding systems and production.

1. Introduction
Vaccinium ssp. are a species from the family Ericaceae [1]. Armenia spread in different provinces: Vaccinium uliginosum (incl. in Red Book of Armenia) - Syunik; Vaccinium myrtillus - Lori, Tavush. Grows in upper mountain, subalpine and alpine zones (2800-3300 m above sea level), meadows, forests [2].

Blueberries are traditionally propagated via stem cuttings [3], [4].

The main goal of this work was the introduction in vitro culture of wild Armenian blueberry plants, the study of the phytohormonal composition effect on plant growth and the application of the stratification method.

2. Materials and methods
Research material (seeds from mature fruits) was collected from "Miapor" mountain range expedition on altitude about 2000 m, organized in September 2020. In research work, we analyzed two species of wild blueberry: Vaccinium myrtillus and Vaccinium uliginosum:

- Vaccinium uliginosum. These are deciduous or evergreen shrubs, reaching a height of up to 30-60 cm. The leaves are arranged consecutively, blue-green color, the edge of the leaf lamina is entire, up to 1,1cm. Shoots are brown. The blossoms are 1- or 4-sectional, collected in axil bunches. The corolla is bulblobell-shaped, the stamen is 8-10 in number, and the ovary is
positioned low with 4-5 carpels. The fruit is a juicy blue with small seeds. Vaccinium uliginosum included in red book of flora Armenia.

- Vaccinium myrtillus. These are deciduous or evergreen shrubs, reaching a height of up to 20-40 cm. The leaves are arranged consecutively, light green color, oviform, up to 3.0 cm. Shoots are green. The blossom is single. The corolla is bulb to bell-shaped, the stamen is 8-10 in number. The fruit is a juicy blue-black with small seeds.

- These plants thrive in acidic soil and require ample sun light. The shrubs are medium-sized and bear clusters of blue to purple fruits with ashen coatings. The berries are delicious with sweet, tart, tangy taste. The ripe berries harvested in autumn, ideally from September to October.

Introduction into in vitro culture organized according to the common methods.

Explants (seeds) were isolated in culture tubes with each containing 15mL of modified culture medium. The medium in all experiments was supplemented with Murashige and Skoog (MS) vitamins, 30 g L−1 sucrose, 0.1 g L−1 myo-inositol, and 6 g L−1 agar. Media pH was adjusted to 5.2 before autoclaving at 120 °C and 1.5 atm.

The steps and conditions of the research work:

- Preparation of plant material. With aim of increasing seed germination have been used cold stratification of seeds. The stratification conditions were determined. A comparative analysis of the germination capacity of stratified seeds and untreated seeds was carried out.

- Micropropagation or microclonal reproduction. The process of micropropagation divided into following three steps.

  **Initiation.** The objective of this stage is to achieve an aseptic culture. Determined the blueberry seed sterilization conditions for introduction into in vitro culture. Sterilized seeds cultured in Murashige and Skoog medium without hormones and vitamins and had been preserved at 26 °C and kept in the dark for 7-10 days in thermostat.

  **Multiplication.** For multiplying of material have been used microcutting technique. The micropropagation of blueberries investigated depending on the origin of genotypes and the hormonal composition of the nutrient medium: kinetin 0.1-0.6 mg/l and 6-Benzylaminopurine (6-Ba) 0.1-1.0 mg/l.

  **Rooting.** As a basis mediums were served - Murashige and Skoog (MS). The pH of all media was adjusted to a final pH of 4.9-4.8 with HCl before autoclaving. Researched the effect of different concentration and composition of the phytohormones – Indole-3-acetic acid (IAA) 0.5-1.5 mg/l, Naphthaleneacetic acid (NAA) 0.5-1.5 mg/l, Indole-3-butyric acid (IBA) 0.5-1.5 mg/l.

All cultures used for evaluation of media, growth regulators, and rooting were maintained in a culture cabinet under a 16-h photoperiod with temperature of 25 ± 2 °C. All the stages of the research were repeated three times.

Statistical analysis.

The statistical analysis of seeds germination in vitro conditions, blueberry reproduction rate and roots formation from shoots calculated by IBM SPSS 2011 (Statistical Package for the Social Science (SPSS) statistical software) (version 20.0).

3. Results and Discussion

**Cold stratification effects on the germination of seeds.** According to many authors, cold stratification break the seeds’ rest period, so they are ready for planting [13]. For analysing of cold stratification effect on seed germination in our study we used per 200 pcs for each stratified and unstratified samples. Stratification of blueberry seeds consisted of storage at 4-5°C for 1, 2, 4, 6 and 8 weeks and unstratified seeds had been preserved at 26 °C and kept in the dark for 7-10 days in thermostat. Seed germination and disinfection for both stratified and unstratified treatments was in the same condition.
Disinfection was carried out in a laminar flow chamber as follows: rinsed with ethanol 96% for 20 s, dipped in a solution of 12% hydrogen peroxide for 10 min. Research results are given in the table 1.

**Table 1.** Seeds germination *in vitro* conditions after cold stratification.

| Seeds storage, weeks | Seeds germination *in vitro* conditions, % |
|----------------------|---------------------------------------------|
|                      | *Vaccinium myrtillus* L. | *Vaccinium uliginosum* L. |
|                      | After cold stratification | Unstratified seeds | After cold stratification | Unstratified seeds |
| 1                    | 1.2±0.35                  | 0.0                 | 0.0                       | 0.0                 |
| 2                    | 1.6±0.14                  | 0.0                 | 1.1±0.02                  | 0.0                 |
| 4                    | 45.2±0.46                 | 12.2±0.46           | 40.1±0.57                 | 8.3±0.15            |
| 6                    | 65.6±1.20                 | 15.2±0.79           | 62.2±0.96                 | 50.3±1.24           |
| 8                    | 88.3±1.71                 | 80.1±1.95           | 74.2±0.86                 | 65.2±1.03           |

The cold stratification of *Vaccinium myrtillus* seeds for 8 weeks contributed for obtaining the highest number of germinated seeds 88.3%, whereas without stratification the germination percentage was 80.1, as can be seen from the data in table 1. The similar situation was observed during seed germination of *Vaccinium uliginosum* – in the case of stratification 74.2%, with normal germination – 65.2%.

The smallest result of 1.2-1.6% was obtained by stratification of Vaccinium myrtillus seeds, for 1-2 weeks and a zero result without stratification. The Vaccinium uliginosum seeds were germinated only in the 2nd week, the percentage was 1.1, while without stratification no seed was germinated. In figure 1 show stages of in vitro germination of blueberry Vaccinium myrtillus seeds.

**Effects of nutrient media and hormonal composition on shoot multiplication.** The nutrient media have significant impact on multiplication and growth of wild blueberry. For the multiplication phase MS basal nutrient medium was modified supplemented 6-Ba 0.1-1.0 mg/l and Kinetin 0.1-0.6 mg/l. Studies have shown that the highest reproduction rate for both species of 5.3 – 6.2 was observed on nutrient media containing 0.6 mg/l 6-Ba. The number of formed plants decreased to 0.9-1.0 with an increase in concentration to 1 mg/l. The low reproduction rate of 0.1 -2.5 was observed when kinetin was adds as cytokine. Kinetin had the worst performance (table 2). The same results was found from authors where law concentrations of 6-Ba did not show any significant response [14-18]. On MS₀ of absence of growth regulators the reproduction rate was very law.

**Table 2.** 6-Ba and Kinetin concentration influence on blueberry reproduction rate.

| Growth regulators, mg/l | *Vaccinium myrtillus* | *Vaccinium uliginosum* |
|-------------------------|-----------------------|------------------------|
| MS₀                     | 0.0                   | 0.03±0.0043            | 0.025±0.001            |
| 6-Ba                    | 0.1                   | 0.5±0.03               | 0.2±0.01               |
|                         | 0.2                   | 1.2±0.14               | 0.7±0.023              |
|                         | 0.4                   | 1.8±0.15               | 1.2±0.07               |
|                         | 0.6                   | 6.2±0.26               | 5.3±0.16               |
|                         | 0.8                   | 4.2±0.18               | 2.5±0.08               |
|                         | 1.0                   | 0.9±0.07               | 1.0±0.02               |
| Kinetin                 | 0.1                   | 0.7±0.05               | 0.5±0.03               |
|                         | 0.2                   | 2.5±0.08               | 1.3±0.09               |
|                         | 0.4                   | 0.2±0.01               | 0.3±0.02               |
|                         | 0.6                   | 0.1±0.06               | 0.0                   |

LSD₀.₀₅ 0.6
Rooting. The rooting of the obtained microcuttings is an important stage of micropropagation. The choice of the rhizogenesis – auxin inducer and the selection of its optimal concentrations have decisive (value) factor in rooting. Rooting can also be induced in the shoot proliferation medium without plant growth regulators or in medium containing IBA or NAA [15].

In this study we examined the effect of IAA, NAA and IBA on induction of rhizogenesis on wild blueberry. The shoots were divided in microcuttings with one or two axillary buds and placed them on MS media, supplemented with various concentration of IAA 0.5-1.5 mg/l, NAA 0.5-1.5 mg/l, IBA 0.5-1.5 mg/l.

Percentage of the rooted plants as well as the other rooting parameters such as number and length of roots, were determined after 20 days (table 3).

In our study on MS0 medium without grow regulators the root formation was occurred, but the percentage of rooting, number and length of roots was law.

The use of IAA in a concentration of 0.5 mg/l contributed to the rooting of 56.2% of shoots, with the number of roots – 3.0 pcs/expl. and root length 35.2mm. The IAA concentration increase to 1.0 and 1.5 mg/l not only didn't contribute rooting, but on the contrary, inhibition of the root formation process and callus formation were observed.

In use of NAA the best result obtained at a concentration of 1.5 mg/l, when indicators were: rooting – 67.2%, number of roots – 5.5 pcs/expl., root length – 33.6 mm.

The addition of 1.0 – 1.5 mg/l IBA to the medium contributed to the highest production of rooted shoots 88.9 and 96.1% with the largest number of roots per explant – 5.7 and 6.0 and the roots length are 48.2 and 54.6, as can be seen from the table 3.

Table 3. Influence of auxins IAA, NAA and IBA on roots formation from shoots.

| Growth regulators | Concentration, mg/l | Rooting, % | The number of roots pcs/expl | Root length, mm |
|-------------------|---------------------|------------|-----------------------------|----------------|
| MS0               | 0.0                 | 15.8±0.34  | 1.8±0.02                    | 4.3±0.33        |
|                   | 0.5                 | 56.2±1.52  | 3.0±0.068                   | 35.2±1.14       |
| IAA               | 1.0                 | 16.2±0.38  | 3.0±0.16                    | 15.6±1.08       |
|                   | 1.5                 | 10.1±0.03  | 3.9±0.62                    | 17.6±1.83       |
|                   | 0.5                 | 27.7±0.53  | 3.5±0.54                    | 13.2±0.71       |
| NAA               | 1.0                 | 53.1±0.86  | 3.7±0.57                    | 33.0±0.91       |
|                   | 1.5                 | 63.5±1.03  | 5.7±0.53                    | 43.2±0.95       |
|                   | 0.5                 | 84.2±1.11  | 5.5±0.79                    | 35.1±0.89       |
| IBA               | 1.0                 | 88.9±1.64  | 5.7±0.76                    | 48.2±1.05       |
|                   | 1.5                 | 88.2±1.68  | 6.0±0.83                    | 60.1±1.42       |

1 V. myrtillus L.; 2 V. uliginosum L.

4. Conclusion
These studies reveal that optimum conditions for in vitro germination of wild blueberry Vaccinium myrtillus and Vaccinium uliginosum seeds were: cold stratification 4-5 °C for 8 weeks, where in the percentage of germinated seeds was 74.2 – 88.3%. Phytohormone ratios are crucial for determining
cell development fate during in vitro growth regulators should always be taken into account in order to obtain best results. MS medium supplemented 6-Ba 0.6 mg/l added increases shoot multiplication efficiency (5.3 – 6.2) of wild blueberries in vitro.

The addition of 1.0 – 1.5 mg/l IBA to the medium contributed to the highest production of rooted shoots 88.9 and 96.1% with the largest roots number per explant – 5-7, the roots length is 48.2 and 54.6 mm.

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