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Research Article

Micropropagation of bog blueberry (*Vaccinium uliginosum* L.) distributed in Bulgaria

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Abstract

The aim of the present study is to develop a system for micropropagation of bog blueberries. They are distributed from 1700 to 2500 m above sea level in the mountains Rila, Pirin, Western and Central Balkan Mountain, Vitosha, Osogovo, Western Sredna Gora and Western and Middle Rhodopes. An approach for the statistical optimization of micropropagation media under laboratory conditions has been applied for the first time. Sterile explants were introduced *in vitro* on WPM, enriched with 3 mg.l^{-1} 2-iP and pH 4.2. To optimize proliferation, nine variants of WPM were tested with the addition of growth regulators (zeatin and 2-iP) in varying concentrations (1.5 - 3.5 mg.l^{-1}), organic additives (casein hydrolisate and myo-inositol and pH values (4.3 - 4.8). The best result regarding the reproduction coefficient was found on M1 nutrient medium (3.77 pcs/explant), enriched with 3.5 mg.l^{-1} zeatin and 2-iP, 250 mg.l^{-1} casein hydrolisate, pH 4.8. The largest average length of shoots was reported on the modified medium M9 with 2.63 cm. The resulting micro shoots were rooted on WPM nutrient medium, halved macrosol content and different levels of IAA (0.5 - 1 mg.l^{-1}), myo-inositol and pH. The highest percentage of rooting was found on R8 nutrient medium with 56.7%.

Keywords

Vaccinium uliginosum L., bog blueberry, micropropagation, tissue culture, 2-iP, zeatin, IAA, casein hydrolisate

Abbreviations

AC – activated charcoal; CH – casein hydrolysate; IAA – Indole-3-acetic acid; IBA – indole-3-butyric acid; MI – myo-inositol; WPM – woody plant medium; zeatin – 4- Hydroxy-3-methylbut-2-enylamino purine; 2iP - N6 – isopentenyladenine

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Introduction

Blueberry is a widespread crop around the world, but the best soil and climatic conditions for its growing are in the United States, Europe, Australia and New Zealand. It belongs to the family Pyrenees (*Ericaceae*) and the genus *Vaccinium*, which is one of the richest in species and includes about 450-500 representatives. The bog blueberry (*Vaccinium uliginosum* L.) is a representative of the Bulgarian flora, with stem height up to 50 cm, growing on stony and rocky places. Its natural populations in Bulgaria are located in the high parts of the mountains. Blueberry is a plant species, extremely rich in biologically active substances of interest to the medical and pharmaceutical industries.

In recent decades, *in vitro* technology has been the traditional method of propagation of plant species. The concept of properly understanding the blueberry micropropagation technology involves the choice of the appropriate basal nutrient medium and growth regulators. The tissue culture method in bog blueberries will help to study, characterize and select its valuable genotypes (Kim et al. 2009).

Some researchers recommend different variants of a modified and agarized WPM nutrient medium for accelerated blueberry reproduction (Lloyd and McCown 1980; Wolfe et al. 1983; Reed and Esquivel 1991; Gajdošová et al. 2006; Kaldmäe et al. 2006; Meiners et al. 2007; Georgieva and Kondakova 2008; Georgieva 2013; Georgieva et al. 2016; Hung et al. 2016), whereas other authors suggest AN nutrient medium (Anderson 1975), which also has a reduced ammonium and nitrate content (Grout et al. 1986; Ostrolucká et al. 2004; Gajdošová et al. 2006). Debnath and McRae (2001a, b) and Debnath (2005a, b, c) successfully micropropagated *V. macrocarpon*, *V. vitis-idaea* L. on a modified MS nutritional medium (Debnath and McRae, 2001) by reducing the amount of macro salts, micro salts, and vitamins and replacing calcium chloride with calcium gluconate. Cüce and Sökmen (2017) compared the three main nutrient media, such as WPM, MS (Murashige and Skoog 1962) and AN for accelerated reproduction of *Vaccinium uliginosum* L. and found that WPM is the most effective as the main medium for multiplication.

The aim of the present study is to develop an experimental protocol for *in vitro* propagation of blueberry (*Vaccinium uliginosum* L.) with optimal efficiency in the use of growth regulators, such as 2-iP, zeatin, IAA and organic additives, such as casein hydrolisate, myo-inositol and pH of agarized WPM nutrient medium.

Materials and Methods

Plant material. *V. uliginosum* was chosen as the objective of the present scientific experiment. Natural populations of bog blueberries originate in the Western Rhodopes near Perelik hut, 1960 m above sea level, GPS: 41°36'28.6"N 24°35'46.9"E

The scientific experiment was conducted in the laboratory for micropropagation of small-sized fruit species at RIMSA in Troyan.

Photo 1. Natural habitat of bog blueberries on Perelik peak

Sterilization of plant material and introduction into *in vitro* culture. Leafless two-bud softwood cuttings 1.5-2 cm long were used as initial explants from *in vivo* plants from the natural habitats of Perelik peak.

Nutrient medium for the introduction of bog blueberries is WPM, enriched with 3 $mg.l^{-1}$ 2-iP. Blueberry is an acidophilic species and the acidity of the medium is 4.2 and 4.8.

Sterilization of plant material included:

- Flushing under running water for one hour:
- Surface sterilization in laminar box conditions:

- 70 % C_2H_5OH for 30 s.

- 0,1% $HgCl_2$ for 3 min.

Rinsed three times with sterile distilled water.

The microcuttings introduced into *in vitro* culture of the genus *Vaccinium* representatives are grown

in growth chambers under controlled conditions, such as temperature 2±25°C, photoperiod 16/8 day/night and illumination of 2000-3000 lx. The incidence of subcultivation is 30 days.

In the *in vitro* propagation and rooting process, a basal WPM nutrient medium with varying concentrations of growth regulators, organic additives and pH values was used, as they are shown in Table 1 and Table 2. Explants separated from pre-selected bog blueberries branches were cultivated. The scheme of the parameters according to which the researches on the processes of micropropagation and rooting are performed are reflected in Table 1- and Table 2.

Table 1. Concentration of growth regulators used, casein hydrolysate and pH before autoclaving

Variants of nutrient	Basal medium	Zeatin mg.l^{-1}	2-iP mg.l^{-1}	Casein hydrolisate mg.l^{-1}	pH
M1	WPM	3.5	3.5	250	4.8
M2	WPM	3.5	3.5	250	4.3
M3	WPM	3.5	3.5	150	4.8
M4	WPM	3.5	1.5	250	4.8
M5	WPM	2.0	3.5	250	4.8
M6	WPM	2.0	1.5	150	4.3
M7	WPM	2.0	1.5	150	4.8
M8	WPM	2.0	1.5	250	4.3
M9	WPM	2.0	3.5	150	4.3

Table 2.
Composition of nutritional medium tested during the rooting phase of bog blueberries

Variants of nutrient medium	1/2WPM	IAA mg.l^{-1}	Myo-inositol mg.l^{-1}	pH
R1	1/2WPM	1.0	500	4.8
R2	1/2WPM	0.5	500	4.8
R3	1/2WPM	1.0	250	4.8
R4	1/2WPM	1.0	500	4.3
R5	1/2WPM	0.5	250	4.3
R6	1/2WPM	0.5	250	4.8
R7	1/2WPM	0.5	500	4.3
R8	1/2WPM	1.0	250	4.3
R9	1/2WPM	0.75	375	4.55

Adaptation. Well-developed and rooted *in vitro* bog blueberry plants are adapted in acidic peat substrate under polyethylene, with high air humidity of 100%, which gradually decreases, and a temperature of 20-22°C for 40 days. Air humidity was controlled by gradually opening the polyethylene.

Data reliability was confirmed by Duncan's multiple comparison test.

Results and Discussion

Although *in vitro* propagation protocols of bog blueberry species have been developed at many laboratories, the individual components have not been extensively studied. The growing conditions, the main nutrient medium, the type and concentration of the growth regulators used, the active acidity of the medium, etc., are of primary importance for the success of the accelerated reproduction technology. Laboratory-grown bog blueberries behave differently from commercial cultivars (Georgieva et al. 2016).

According to Cüce and Sökmen (2017), genus *Vaccinium* is more variable than other members of the Ericaceae family, which requires the development of individual protocols to optimize the individual parameters of the micropropagation process, resulting in time savings and reduced production costs.

In the current model system for clonal propagation of *Vaccinium uliginosum* L., nine agarized nutrient media (M1 - M9) based on WPM were compared with different combinations and concentrations of cytokinins, casein hydrolisate and pH in order to obtain more seedlings from one axillary bud.

On Fig. 1 and Fig. 2 are presented the results of the impact of nutrient media, growth regulators and vitamin supplements on the number and length of newly formed shoots. The data analysis from the scientific experiment shows that the average proliferation coefficient of the tested variants in bog blueberries varies from 2.48 (M3) to 3.77 (M1) number of explants (Fig. 1). The highest average values of the reproduction coefficient were registered on M1 (3.5 mg.l^{-1} zeatin and 3.5 mg.l^{-1} 2-iP, 250 mg.l^{-1} casein hydrolisate and pH 4.8). For bog blueberries, the best results are found at a ratio of 1:1, zeatin: 2-iP.

The average length of *in vitro* shoots varies from 2.63 (M9) to 1.10 (M2), as no definite interconnection was observed between their length and the number of tested nutrient media.

Georgieva and Kondakova (2021) studied the micropropagation ability of five cultivars of highbush blueberry and found the highest multiplication factor in the Bluegold cultivar (4.4 shoots/explant) of WPM, containing 3 mg.l^{-1} zeatin, 2 mg.l^{-1} 2-iP pH 4.2.

A key element in *in vitro* technology in bog blueberries is the right choice of cytokinins, appropriate combinations and concentrations. The most commonly used growth regulators in blueberry micropropagation are zeatin and 2-iP. There are numerous scientific reports that convincingly prove that zeatin is the more effective cytokinin and is more suitable for multiplication in *Vaccinium* sp. (Debnath and McRae 2001b; Ostrolucká et al. 2002; Ondrušková et al. 2003; Ostrolucká et al. 2004). Cüce and Sökmen (2017) recorded the largest length (40.02 mm) and number of shoots (3.73) in blueberry when enriching the nutrient medium with 2.0/0.1/0.2 mg.l^{-1} zeatin/IBA/GA_3

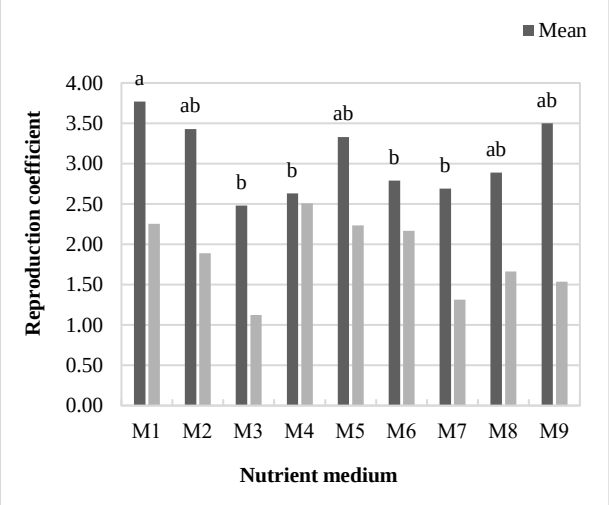

Figure 1. Reproduction coefficient of bog blueberries on different nutrient media

* Duncan's multiple comparison test was conducted. Means followed by the same letters are not significantly different at P<0.05

Although auxins and cytokinins are major groups of phytohormones used in tissue cultures, there are many other growth-promoting substances that can be used to stimulate cell division and differentiation *in vitro*.

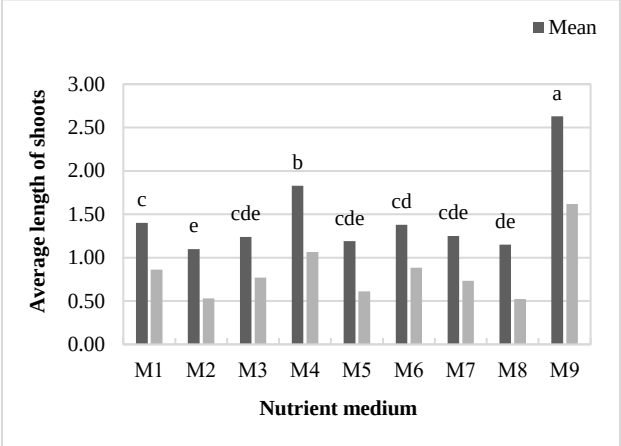

Figure 2. Average length of shoots (cm) of bog blueberries on different nutrient media

* Duncan's multiple comparison test was conducted. Means followed by the same letters are not significantly different at P<0.05

The use of organic additives such as casein hydrolisate and myo-inositol in the nutrient medium are essential for stimulating many biochemical reactions in the plant organism.

Rooting of *Vaccinium uliginosum* L. was assessed depending on the concentration of IAA, myo-inositol and pH of the nutrient medium. Blueberry is poor in endogenous hormones and enrichment of the environment with appropriate auxin and organic supplement is a prerequisite for the formation of a good root system (Georgieva and Kondakova 2008). IAA was tested in the experiment at levels of 0.5-1 mg.l^{-1}, which gave good results in previous studies with wild black blueberries and cranberries and different cultivars of highbush blueberry (Georgieva and Kondakova 2008; Georgieva et al. 2016; Georgieva and Kondakova 2021). The use of myo-inositol as an organic additive has been dictated by our previous studies, in which it promotes root induction in other representatives of genus *Vaccinium* (Georgieva et al. 2016).

All tested variants showed rooting (Fig. 3), ranging from 20% to 56.7%. The best results were achieved on R8 nutrient medium containing 1 mg.l^{-1} IAA, 250 mg myo-inositol and pH 4.3, followed by R4 (1 mg.l^{-1} IAA, 500 mg.l^{-1} myo-

inositol and pH 4.3). Satisfactory rooting was performed on agarized media, such as R2 (20%), R1 (26.7%) and R6 (26.7%). In our previous studies on highbush blueberry, the best rooting was achieved in *in vitro* conditions of Brigitta blue cultivar (81.8%) on medium containing 0.5 mg.l^{-1} IAA (Georgieva and Kondakova 2008).

In other our studies (Georgieva and Kondakova 2021) the rooting of five commercial highbush blueberry cultivars (Bluecrop, Bluegold, Blujay, Spartan, Patriot) was observed, recording the highest percentage of rooting (33.3%) and the largest average number of roots (3.9 pieces) in Spartan and Patriot genotypes.

Erst et al. (2018) enriched Anderson's nutritional medium with IBA and IAA and observed an increase in the rooting rate and quality of blueberry roots. In their study, similar percentages of rooting of *Vaccinium uliginosum* L. were obtained at the same levels of both auxins.

Cüce and Sökmen (2017) compared the impact of different types and levels of auxins (IBA, IAA and NAA) with or without active charcoal on the rooting of *Vaccinium uliginosum* L. and found that NAA in combination or in the absence of AC did not induce *in vitro* rooting. Active charcoal applied alone had no stimulatory effect on rooting. The authors obtained the highest rooting percentage on WPM nutrient medium (18.89%) supplemented with 0.5/1.0 mg.l^{-1} IBA/AC, which prove the specificity in the effect of the individual type of auxin on *Vaccinium uliginosum* L.

The quality of the root system was determined by the average length and average number of roots of the *in vitro* plants. Average root length in the indicated experimental design in blueberries ranged from 0.6 cm on variant R2 to 0.82 cm – R6. The average number of roots was in the range of 1.67 (R2) to 3.5 (R5).

Adaptation. Optimizing the blueberry rooting protocol will contribute to increasing plant survival under *ex vitro* conditions (Erst et al. 2018).

Adaptation of the *in vitro* blueberry plants in a greenhouse was successful and 92% of them survived. When they reach a height of 7-10 cm, they were transferred to larger containers filled with a mixture of peat and perlite (2:1). *Ex vitro* plants were sufficiently viable and normally developed.

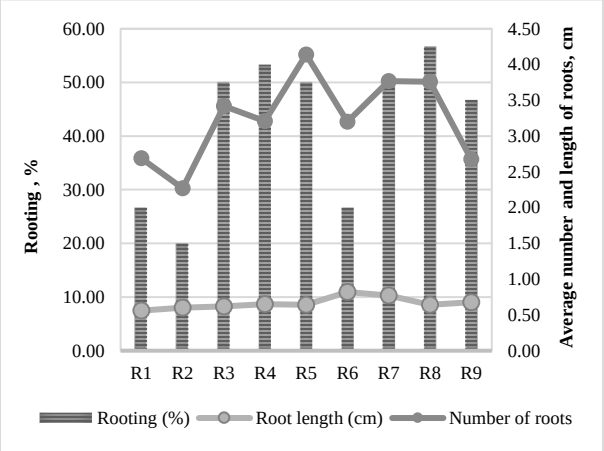

Figure 3. *In vitro* rooting percentage (%) of bog blueberries on different nutrient media

* Duncan's multiple comparison test was conducted. Means followed by the same letters are not significantly different at P<0.05

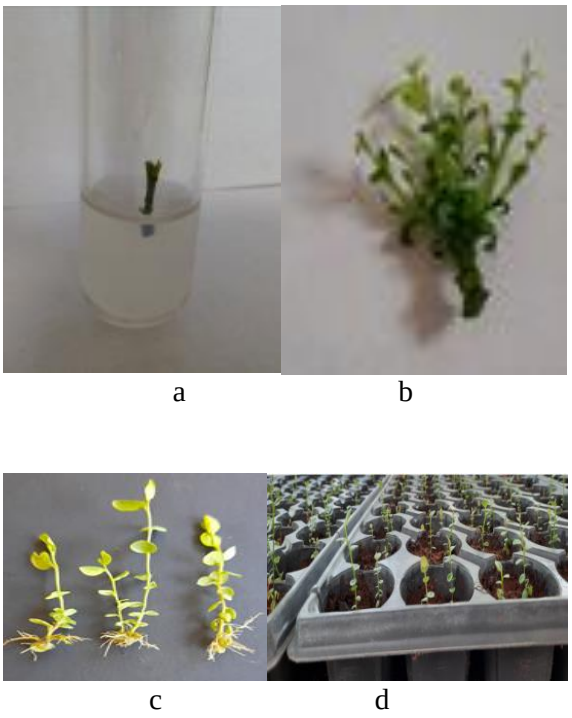

Figure 4. Scheme of *in vitro* propagation introduction (a), multiplication (b), rooting (c) and adaptation (d) of bog blueberries

Conclusions

In conclusion, the results presented in this scientific experiment show that the *in vitro* response is specific depending on the type and level of cytokinin used, the organic supplement and the active acidity of the culture medium.

Vaccinium uliginosum L. plant genotypes prove that the combination of zeatin 2-iP in a ratio of 1:1 on M1 agar nutritional medium was the most suitable for accelerated propagation of *Vaccinium uliginosum* L.

The most suitable nutrient medium for accelerated reproduction of blueberry was M1 including the combination of the growth regulators zeatin and 2-iP in a ratio of 1:1.

IAA had a positive effect on the induction of root formation in bog blueberry. Higher levels of IAA had a stimulatory effect on rhizogenesis of *Vaccinium uliginosum* L. Rooting was most successful on R8 culture medium containing 1 $mg.l^{-1}$ IAA, 250 mg myo-inositol and pH 4.3.

The optimization of the individual parameters of the micropropagation process of blueberry will help to preserve for future studies of this valuable ecotype of *V. uliginosum* in territory of Bulgaria.

The transfer of the micropropagated plants of *Vaccinium uliginosum* L. in a soil substrate was successful and 92% of them survived.

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