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RESEARCH ARTICLE
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Optimization of the Technological Cycle of Clonal Micropropagation of *Vaccinium Praestans* Lamb

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ABSTRACT

The study evaluated clonal micropropagation of Kamchatka bilberry (*Vaccinium praestans* Lamb.) using three forms collected from their natural range (Korsakovskaya, Iturupskaya, and Khabarovskaya). During surface sterilization, 100 explants per treatment (form × sterilant × exposure) were tested in triplicate. Subsequent stages used 10 plants per treatment in triplicate with factorial designs as follows: (i) shoot proliferation—medium strength × cytokinin level × biostimulant; (ii) rooting—auxin type × auxin dose × biostimulant. Data were analyzed by factorial ANOVA, and means were separated by the least significant difference at $\alpha = 0.05$ ($LSD_{0.05}$). The highest survival rate of *V. praestans* explants (92–97%) at the stage of introduction into *in vitro* culture was found when they were sterilized with 0.2% silver nitrate solution at the exposure time of 10 minutes. The highest values of number of *V. praestans* microshoots in *in vitro* culture at the stage of proliferation were observed in options with the 1/2 Woody Plant Medium, the maximum length of microshoots was observed upon addition of 1.0mg L^{-1} 6-benzylaminopurine + 0.1mL L^{-1} HB-101 preparation. The rooting percentage of *V. praestans* explant *in vitro* culture on 1/2 WPM medium with the addition of both IBA and IAA at concentrations of 1.0-2.0mg $L^{-1}$was 80-96%. Increasing the concentration of 6-benzylaminopurine promoted a 1.2–1.5-fold increase in the *V. praestans* microshoots *in vitro* and a slight decrease in their average length. The maximum number and length of *V. praestans* roots *in vitro* at the stage of rhizogenesis were observed on the 1/2 Woody Plant Medium with addition of 2.0mg L^{-1} indole-3-butyric acid + 0.1mL L^{-1} HB-101 preparation. The highest survival rate of *V. praestans* regenerated plants (90–96%) during adaptation to non-sterile conditions *ex vitro* was noted when transplanted in May on peat + zeolite 1:1, peat + river sand 1:1, peat + perlite 3:1. The survival rate of the *V. praestans* plants obtained by the method of clonal micropropagation 14 days after transplantation in open ground conditions of Kostroma region, Russia was 81–90%.

Keywords: Forest berry plants, Growth regulators, Kamchatka bilberry, *In vitro*, Nutrient medium, Woody Plant Medium, 6-BAP, IBA/IAA, Sphagnum mulching, Acclimatization

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INTRODUCTION

Recently, there has been increasing interest in growing wild berry plants of the genus *Vaccinium* L., family *Ericaceae*, which are highly valuable for food, medicine, and ornamental purposes. The subfamily *Vaccinioideae* of family *Ericaceae* contains more than 4 thousand species, including the genus *Vaccinium* L. which contains about 450-500 species common in Europe, Southeast and Central Africa, Asia, North and Central America (Luby et al., 1991; Tundis et al., 2021; Martău et al., 2023). Berry plants of the genus *Vaccinium* are among the best dietary sources of biologically active compounds, which are of great interest to nutritionists and food industry technologists. The main biologically active substances identified in *Vaccinium* spp. include anthocyanins (cyanidin, malvidin and delphinidin), flavonoids (quercetin, isoquercetin and astragalin), phenolic acids (gallic, P-coumaric, cinnamic, syringic, ferulic and caffeic acids), and iridoids. Numerous scientific studies of the fruits of *Vaccinium* spp. confirm their high content

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of biologically active substances with pronounced antioxidant, antimicrobial and anti-inflammatory properties, helping in the fight against diabetes, obesity, cancer, atherosclerosis, rheumatoid arthritis, neurodegenerative and cardiovascular diseases, etc. In addition, it is believed that *Vaccinium* spp. plants, especially those with a high content of polyphenols such as anthocyanins, are able to inhibit and initiate apoptosis in cancer cells. Scientists have described the inhibition of tumor growth, angiogenesis and metastasis when using extracts of *Vaccinium* spp. fruits (Chehri et al., 2022; Dunford, 2022; Albert et al., 2023; Alsharairi, 2024).

Consumer demand for fruit products of forest berry species with high nutritional and medicinal value is increasing. One of these species is the Kamchatka bilberry (in Russia this plant is known as "krasnika" and "klopovka") — *Vaccinium praestans* Lamb. It is a shade-tolerant shrub with large leaves, sweet-sour fruits. *V. praestans* is an endemic species whose range spans the Pacific coastal regions of the Russian Far East and parts of the Japanese archipelago. It is mesophytic and acidophilic, generally cold-hardy yet notably susceptible to late spring frosts (Krasikova, 1987; Nechaev & Nechaev, 2012). *V. praestans* fruits are rich in flavonoids and other P-active compounds, ascorbic and benzoic acids, tannins, trace elements. Ripe fruits of *V. praestans* accumulate up to 192mg 100 g^{-1} FW of vitamin C. Also, organic acids, fiber, seven essential amino acids and microelements (Cu, Co, Mn, Zn, Cr) were found in *V. praestans* berries. The amino acid content in *V. praestans* berries averages about 8.42%. Gutamic acid (19.0% of the total amino acids), aspartic acid (10.1%) and arginine (9.1%) predominate among the replaceable amino acids in the fruits of in *V. praestans*. The prevailing essential amino acids of the berries of *V. praestans* are leucine (7.2% of the total amount), lysine (5.5%) and valine (5%). The amino acids of the berries of *V. praestans* have a pronounced biological effect on various systems of human organs. To date, the antioxidant activity of polyphenolic compounds of *V. praestans* has been proven, the most important of which are flavonoids and anthocyanins (Salikova et al., 2021). Juices, fruit drinks, jams are prepared from the fruits, berries are used in confectionery production and in the preservation of products. Extracts from berries can be used in the treatment of many diseases (Plaksen et al., 2014). The results of this species introduction in the Moscow region showed the prospects of growing *V. praestans* in the conditions of the European part of Russia middle zone (Smirnov, 2003).

Many representatives of the genus *Vaccinium* can be successfully grown on an industrial scale in areas of depleted peat deposits and drained bogs, including in northern regions (Noormets et al., 2003; Tyak et al., 2016). But the use of traditional methods of propagation (by seeds, green cuttings, woody cuttings, root shoots) is not very effective in the industrial cultivation of berry plants, since a small yield of plants from one parent is obtained. To accelerate the production of healthy and high-quality planting material in large quantities, the method of clonal micropropagation should be used. The likelihood increases that the planting material obtained in this way will be genetically homogeneous, more resistant to biotic and abiotic environmental factors, and have high potential and stable yields. While axillary shoot culture tends to preserve genotype, somaclonal variation and carry-over effects can occur for which purpose clonal fidelity checks (e.g., SSRs or SNP barcoding) will need to be carried out in the future (Shevelukha, 2015; Majumder et al., 2025). To date, many different studies have been conducted on clonal micropropagation of berry plants from the genus *Vaccinium* such as lingonberry (Stanienė et al., 2002; Paprštein & Sedlák, 2015; Arigundam et al., 2020), blueberry (Debnath, 2009; Bozhiday & Kukharchik, 2014; Hung et al., 2016), cranberry (Debnath & McRae, 2001; Bozhiday, 2018) – at different stages and taking into account varietal characteristics. There are only isolated reports on the introduction of *V. praestans* into *in vitro* culture (Stanienė et al., 2002). We conducted studies on clonal micropropagation of *V. praestans* (Makarov et al., 2021; Chudetsky et al., 2022a, 2022b), however, improvement and optimization of the full technological cycle of cultivation *in vitro* and further adaptation to *ex vitro* conditions for this species are required.

The aim is to study the effect of sterilizing agents on the viability of *V. praestans* explants when introduced into culture *in vitro*, the effect of the culture medium composition, the presence and concentrations of growth–regulating substances in the medium on the morphogenetic potential of regenerating plants during clonal micropropagation, as well as the effect of the substrate and the adaptation period on the survival of plants in non-sterile *ex vitro* conditions.

MATERIALS & METHODS

The objects of research are *V. praestans* plants of 3 natural forms selected in the places of its natural distribution – Korsakovskaya (Korsakovsky District, Sakhalin Oblast, Russia) (N 46.6156, E 142.9585), Iturupskaya (Kurilsky District, Sakhalin Oblast, Russia) (N 45.2946, E 147.8789) and Khabarovskaya (Khabarovsk Krai, Russia) (N 53.0348, E 140.7987) (Fig. 1). These forms are wild-selected clones maintained vegetatively and differ in the ripening time of fruits: Iturupskaya – late, Korsakovskaya – medium, Khabarovskaya – early.

The studies were carried out during the period 2018-2023 using the methods and recommendations already described for clonal micro-reproduction of plants (Shevelukha, 2015). Apical and lateral buds of plants harvested from introduced 3-year-old donor plants in the conditions of the cultivar testing plot in the Kostroma district, Kostroma Oblast, Russia (N 57.7070, E 40.8285) in the 1st decade of May were used as explants (Fig. 2).

Time interval from donor plant collection to laboratory work (culture initiation) is 2 hours. The explants were washed in warm running water, then in distilled water, treated with 70% C_2H_5OH for 30 seconds, then with the main sterilizing solution for 3, 5, 10, 15 and 20 minutes. Various main sterilizing solutions were used to sterilize the plant material: 0.2% silver nitrate, 5% sodium hypochlorite, 10% perhydrol, disinfectants Lysoformin 3000 (Hygiene Plus, Russia) 5% and Nika 2 (Genix, Russia) 0.01%. For each treatment (form × sterilant × exposure), 100 explants in 3

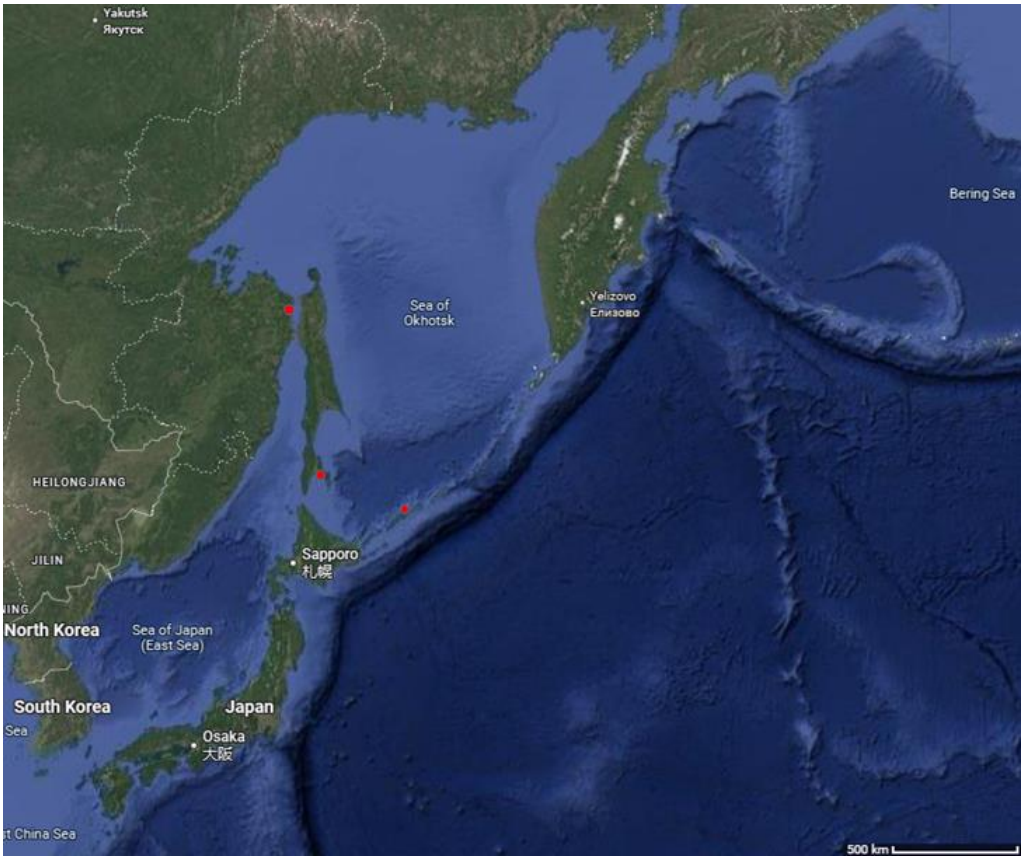

Fig. 1: Locations of selected *Vaccinium praestans* forms.

Fig. 2: Uterine plants of *Vaccinium praestans* (height up to 7 cm) at the experimental site in conditions of Kostroma region, Russia (May 2018) (orig.).

replications were tested. They were cultivated on a on a Woody Plant Medium (WPM) culture medium (Lloyd & McCown, 1980) with sucrose concentration 30g L^{-1}, addition of activated carbon (7g L^{-1}) at initiation stage and in the variants of diluting the mineral salt and vitamins base with bidistilled water by 2 times (1/2 WPM) and 4 times (1/4 WPM) at the shoot proliferation and rooting stages. The pH of the medium before/after autoclaving (headspace 101.325kPa, temperature +120°C, within 18-20 minutes) is 4.5…4.8; medium gel strength 700g cm^{-2}. At the stage of introduction into culture *in vitro*, the explants viability was taken into account for contamination-free and visibly growing at 21–28 days (Fig. 3).

Further, the cultivation of regenerating plants was carried out in test tubes (capacity 13.0mL) in the conditions of light room using fluorescent lamps OSRAM Lumilux L 36W/865 (OSRAM Licht AG, Germany) with color temperature 6500 K, PPFD 72–86µmol m^{-2} s^{-1}, in the ratio red (650-660nm) and blue (440-450nm) light 3:1, at a photoperiod of 16 h light / 8 h dark, air temperature 23–25°C, air humidity 75-80%. At the stage of proliferation of micro-shoots, 6-benzylaminopuryl (6–BAP) was added to the culture medium at concentrations of 0.5 and 1.0mg L^{-1} (Fig. 4) while at the stage of rhizogenesis, indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) at concentrations of 1.0 and 2.0mg L^{-1} was added (Fig. 5).

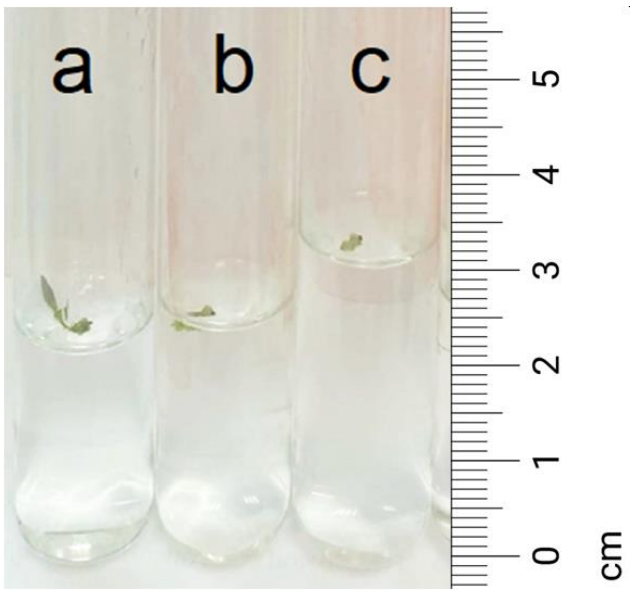

Fig. 3: *Vaccinium praestans* explants at the stage of introduction into *in vitro* culture (orig.); a – Korsakovskaya; b –Iturupskaya; c – Khabarovskaya.

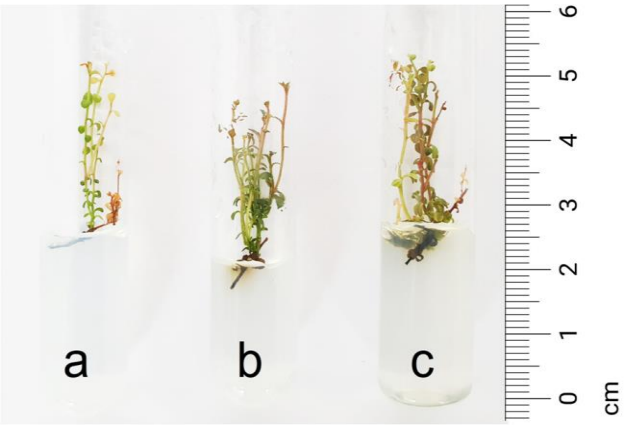

Fig. 4: *Vaccinium praestans* regenerating plant in *in vitro* culture at the stage of proliferation of micro-shoots (orig.); a – Korsakovskaya; b – Iturupskaya; c – Khabarovskaya.

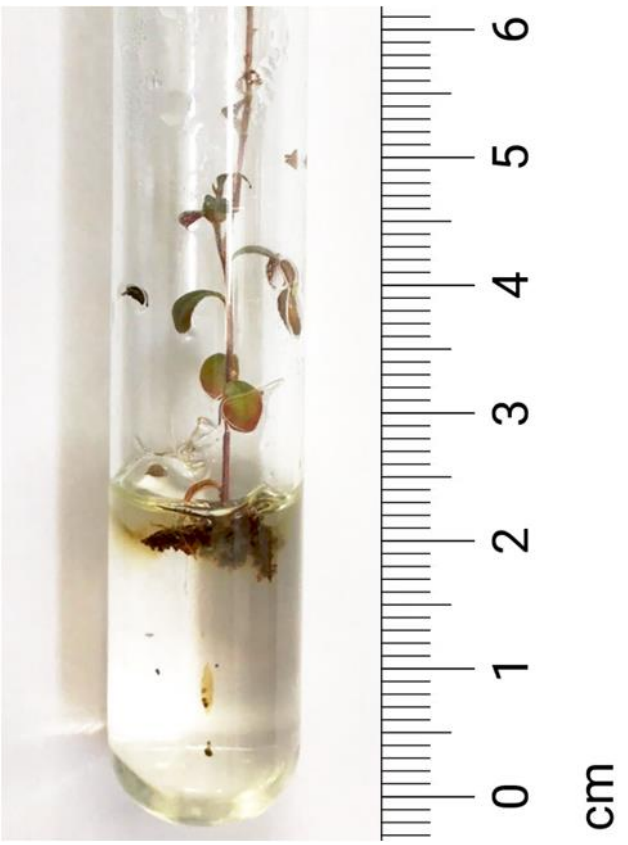

Fig. 5: *Vaccinium praestans* 'Korsakovskaya' regenerating plant in *in vitro* culture at the stage of rhizogenesis (orig.).

At the proliferation and rhizogenesis stages, additives of growth-stimulating drugs Epin-Extra (active ingredient is 24-epibrassinolide 0.025g L^{-1}) (Nest M, Russia) at a concentration of 0.5mL L^{-1} and HB-101 (Flore Co. Ltd., Japan) at a concentration of 0.1mL L^{-1} were used. HB-101 is a commercial plant-extract-derived biostimulant (active ingredients: N – 97.0mg L^{-1}; Na – 41.0mg L^{-1}; Ca – 33.0mg L^{-1}; Si – 7.4mg L^{-1}; Mg – 3.3mg L^{-1}; Fe – 1.8mg L^{-1}) (Loan & Hung, 2019). Epin-Extra (active ingredient is 24-epibrassinolide 0.025g L^{-1}) is a growth regulator and adaptogen with a strong anti-stress effect; it ensures rooting of seedlings during transplantation, accelerates ripening and increases yields, protects plants from adverse conditions, increases resistance to fungal and bacterial infections, stimulates the formation of lateral shoots, reduces the amount of toxins, heavy metals, radionuclides, and excess nitrates in the plant (Anka & Seregina, 2024).

For each regenerated plant, the number and length of micro-shoots and roots, and also multiplication rate and rooting percentage per explant were determined. Three replications (10 plants for each replication) were tested in the experiment. The significant differences between means were evaluated using a two-factorial analysis of variance consistent with the stated factors (on the stage of shoot proliferation: A – cultural medium composition (mineral dilution in cultural medium and cytokinin concentration; B – biostimulant additive; on the stage of *in vitro* rooting: A – auxin type and its concentration; B – biostimulant additive) and the least significant difference for 5 % of the significance level (LSD_{05}) (Dospekhov, 2011). Subculture interval length is 30 days per passage. Total culture age at which multiplication rates were compared across the 7th–9th passages is 6 months.

At the stage of transplanting rooted plants into *ex vitro* conditions from the 1st decade of March to the 3rd decade of May, the following substrates were used: sterile high-moor peat (Agrobalt, Russia) (pH_{KCl} – 2.8...3.5), including mixtures of high-moor peat with river sand (in a ratio of 1:1), vermiculite (3:1), perlite (3:1) and fine-grained zeolite (1:1). The peat was pre-steamed at a temperature of +90°C for 40 minutes using a cover with a humidity of 80-90%. Previously, river sand was washed and calcined at a temperature of +180°C for 2 hours. As components of the substrate, perlite and vermiculite were added to the high-moor peat to improve soil aeration and moisture retention, river sand was added to improve the water permeability of the substrate and air exchange, zeolite was added to improve the chemical and physical characteristics of the soil, air exchange and reduce the need for additional micro–, macroelements and additional acidifying substances. The plants were adapted to *ex vitro* under LED lamps OSRAM Fluora L36/77 T8 (OSRAM Licht AG, Germany) with color temperature 4 000 K, PPFD 165µmol m^{-2} s^{-1}), in the ratio red (650-660nm) and blue (440-450nm) light 3:1, illumination of 8 000lx, using acclimatization domes in the conditions of air temperature +25°C and air humidity 80–90%. Conditions of ambient greenhouse: air temperature +20°C, air relative humidity 70% Plants were sprayed with water in the amount of 10mL per 1 acclimatization dome every 2 hours. At the same time, we laid the experiment with mulching with sphagnum moss. The percentage of viable plants on the total number of tested plants rate was evaluated after 10 days. For each treatment (substrate × transplant time (month) × mulching), 10 plants in 3 replications were tested.

After 60 days of *ex vitro* adaptation, plants were transplanted in open ground conditions on the same variety testing plot in the 3rd ten-day period of May 2023. The planting pattern was 40×15cm, 50pcs. Plants for each form. The survival rate for each form was taken into account as the ratio of the number of surviving plants to the number of transplanted plants 14 days after transplantation.

For statistical processing of experimental data, StatSoft Statistica 10.0.1011 and Microsoft Office Excel 2021 software were used. The statistical tools were 2-way analysis of variance, and least significant difference at the 5% significance level. Data are presented as mean±SE.

RESULTS

A sterile culture of *V. praestans* was obtained for further reproduction. At the stage of introduction of *V. praestans* explants into *in vitro* culture, the most effective sterilizing agent was a 0.2% solution of silver nitrate when used for 10 minutes, where the viability of explants was 92-97%. When treated with Lysoformin 3000 (5%) and Nika-2 (0.01%) at the same exposure, the viability of explants was 1.8–3.6 times less (Table 1). The lowest values (no higher than 32%) were observed with all other sterilizing solutions (sodium hypochlorite (5%), and perhydrol (10%), respectively), where the death of most of the explants from infection was observed. The percentage of vitrification of explants in all variants did not exceed 1%.

At the stage of proliferation, it was revealed that in *V. praestans* regenerating plants, a greater average number of micro-shoots (5.8–5.9pcs. in the studied forms) was obtained on 1/2 WPM culture medium with the addition of 1.0mg L^{-1} of 6-BAP. At the same time 6-BAP 1.0mg L^{-1} increased microshoot number by 23–38% relative to 0.5mg L^{-1} on 1/2 WPM. The number of micro-shoots on the 1/4 WPM culture medium was 9-22% lower than similar concentrations of 6-BAP on 1/2 WPM (Table 2).

An increase in the number of micro-shoots in *V. praestans* regenerating *in vitro* plants was observed on 1/2 WPM culture medium and increasing the concentration of 6-BAP from 0.5 to 1.0mg L^{-1}: by 30-32% for the Korsakovskaya form, by 38-43% for the Iturupskaya form, by 18-26% for the Khabarovskaya form; on 1/4 WPM medium: by 21-64% for the Korsakovskaya form, by 35-38% for the Iturupskaya form, by 6-48% for the Khabarovskaya form.

Table 1: The percentage of viable explants of *Vaccinium praestans* on the total number of tested explants in *in vitro* culture depending on the sterilizing solution and exposure time, %

Sterilizing solution	Exposure time, min				
	3	5	10	15	20
Korsakovskaya					
Sodium hypochlorite 5%	2.00±0.10	7.00±0.24	11.00±0.02	9.00±0.45	7.00±0.57
Perhydrol 10%	3.00±0.11	10.20±0.41	14.50±0.73	25.20±0.14	32.30±2.37
Silver nitrate 0.2%	3.00±0.09	5.00±0.09	92.20±4.24	33.20±0.42	4.00±0.08
Lysoformin 3000 5%	13.00±0.50	16.00±0.44	48.20±1.81	64.10±2.16	77.20±3.13
Nika-2 0.01%	10.00±0.27	17.00±0.33	40.10±0.69	43.20±1.67	32.50±0.70
Iturupskaya					
Sodium hypochlorite 5%	4.00±0.18	7.00±0.31	12.00±0.42	10.00±0.30	8.00±0.59
Perhydrol 10%	3.00±0.04	12.00±0.19	16.00±0.55	23.10±0.20	29.50±2.49
Silver nitrate 0.2%	5.00±0.10	7.00±0.16	97.10±4.78	38.30±1.51	6.00±0.44
Lysoformin 3000 5%	14.00±0.23	21.3±1.04	53.40±1.91	64.40±2.55	82.10±2.39
Nika-2 0.01%	11.00±0.19	19.00±0.75	27.00±1.38	39.10±1.67	31.00±2.65
Khabarovskaya					
Sodium hypochlorite 5%	6.00±0.12	9.00±0.22	13.00±0.39	12.00±0.65	5.00±0.38
Perhydrol 10%	8.00±0.37	14.00±0.66	18.00±0.54	19.00±0.52	28.50±1.98
Silver nitrate 0.2%	24.20±1.15	20.50±0.85	94.20±0.39	32.00±1.03	3.00±0.11
Lysoformin 3000 5%	12.00±0.36	24.10±0.98	57.40±2.55	60.60±2.33	87.00±8.24
Nika-2 0.01%	8.00±0.26	19.20±0.80	44.50±2.17	46.20±1.02	36.10±1.53

Table 2: The number of *Vaccinium praestans in vitro* micro-shoots depending on the concentration of both Woody Plant Medium mineral salts and growth regulators, pcs

Cultural media composition (factor A)		Biostimulant additive (factor B)			Mean value
WPM mineral salt concentration	6-BAP concentration (mg L^{-1})	Without additives (control)	0.5mL L^{-1} Epin-Extra	0.1mL L^{-1} HB-101	
Korsakovskaya					
1/2 WPM	0.5	3.90±0.31	4.50±0.40	4.90±0.42	4.40
	1.0	5.10±0.44	5.90±0.46	6.50±0.59	5.80
1/4 WPM	0.5	2.80±0.20	4.20±0.37	3.90±0.35	3.60
	1.0	4.60±0.38	5.10±0.43	5.90±0.53	5.20
Mean value		4.10	4.90	5.30	-
LSD_{05}, pcs.: A = 1.37; B = 1.13; AB = 1.55					
Iturupskaya					
1/2 WPM	0.5	3.60±0.27	4.10±0.38	4.80±0.41	4.20
	1.0	5.00±0.42	5.80±0.51	6.90±0.61	5.90
1/4 WPM	0.5	3.10±0.25	3.90±0.36	4.40±0.39	3.80
	1.0	4.20±0.37	5.30±0.49	6.10±0.56	5.20
Mean value		4.00	4.80	5.60	-
LSD_{05}, pcs.: A = 1.82; B = 1.41; AB = 2.54					
Khabarovskaya					
1/2 WPM	0.5	4.10±0.36	5.10±0.42	5.30±0.46	4.80
	1.0	5.00±0.43	6.00±0.55	6.70±0.58	5.90
1/4 WPM	0.5	2.90±0.23	4.90±0.43	4.90±0.42	4.20
	1.0	4.30±0.38	5.20±0.47	6.60±0.58	5.40
Mean value		4.10	5.30	5.90	-
LSD_{05}, pcs.: A = 1.59; B = 1.38; AB = 2.23					

When 0.1 ml L^{-1} of HB-101 was added to the nutrient medium, the number of microshoots in the studied forms was higher compared to the control: by 26-39% for the Korsakovskaya form, by 33-45% for the Iturupskaya form, by 29-69% for the Khabarovskaya form. Whereas in the variants with the addition of 0.5mL L^{-1} of Epin-Extra the number of microshoots was higher compared to the control: by 11-50% for the Korsakovskaya form, by 14-26% for the Iturupskaya form, by 20-69% for the Khabarovskaya form.

The reliability of the obtained data on the number of *V. praestans* shoots is confirmed by the results of the ANOVA analysis (F statistic value > F critical value; P<0.05) (Table 3).

With an increase in the concentration of 6-BAP from 0.5 to 1.0mg L^{-1} in the culture medium, a decrease in the mean shoot length of *V. praestans* was observed. While the largest statistically significant differences were revealed only in variants with 1/2 WPM medium, where this indicator in all forms averaged 4.1-4.3cm at a concentration 1.0mg L^{-1}, whereas it was by 86-95% lower than at a concentration of 0.5mg L^{-1}. The addition of 0.1mL L^{-1} HB-101 to the cultural medium contributed to an increase in the mean length of micro-shoots of *V. praestans* plants an averaged by 29-35% relative to the control, whereas addition of 0.5mL L^{-1} Epin-Extra increased the mean shoot length by 12-13% (Table 4).

The reliability of the obtained data on the *V. praestans* shoot length is confirmed by the results of the ANOVA analysis (F statistic value > F critical value; P<0.05) (Table 5).

The highest multiplication rate in all the studied forms of *V. praestans* in *in vitro* culture was noted on the 7th-8th passage, whereas a sharp decrease in the coefficient was observed from the 9th passage. Further studies were carried out on the 1/2 WPM culture medium, since the highest values of the multiplication rate (from 4.0 to 7.0 shoots per explant) of *V. praestans* per regenerating plant were observed on this composition on average according to the experiments (Fig. 6).

The rooting percentage of *V. praestans* explant *in vitro* culture on 1/2 WPM medium with the addition of both IBA and IAA at concentrations of 1.0-2.0mg L^{-1} was 80-86% in the control, 86-92% with the addition of 0.5mL L^{-1} Epin-Extra, and 86-96% with the addition of 0.1mL L^{-1} HB-101 (Table 6).

Table 3: ANOVA results for number of *Vaccinium praestans* shoots in *in vitro culture* (n = 30; α = 0.05)

Source	SS	df	MS	F	p-value	F critical
Korsakovskaya						
Factor A	24.4875	3	8.1625	101.5236	8.98E-14	3.00878657
Factor B	9.045	2	4.5225	56.25	8.73E-10	3.402826105
Factors A×B	1.275	6	0.2125	2.643035	0.041164	2.508188823
Inside	1.9296	24	0.0804			
Total	36.7371	35				
Iturupskaya						
Factor A	24.9	3	8.3	114.1416	2.43E-14	3.008787
Factor B	14.885	2	7.4425	102.3493	1.78E-12	3.402826
Factors A×B	0.795	6	0.1325	1.822141	0.137087	2.508189
Inside	1.7452	24	0.072717			
Total	42.3252	35				
Khabarovskaya						
Factor A	13.79	3	4.596667	32.85876	1.16E-08	3.008787
Factor B	20.285	2	10.1425	72.50253	6.73E-11	3.402826
Factors A×B	2.635	6	0.439167	3.139334	0.020436	2.508189
Inside	3.3574	24	0.139892			
Total	40.0674	35				

Table 4: The mean shoot length of *Vaccinium praestans in vitro* depending on the concentration of both Woody Plant Medium mineral salts and growth regulators, cm

Cultural media composition (factor A)		Biostimulant additive (factor B)			Mean value
WPM mineral salt concentration	6-BAP concentration (mg L^{-1})	Without additives (control)	0.5mL L^{-1} Epin-Extra	0.1mL L^{-1} HB-101	
Korsakovskaya					
1/2 WPM	0.5	3.80±0.25	4.30±0.32	4.70±0.35	4.30
	1.0	1.80±0.16	2.20±0.19	2.60±0.20	2.20
1/4 WPM	0.5	2.50±0.24	2.60±0.25	2.90±0.32	2.70
	1.0	1.50±0.15	1.80±0.16	2.30±0.18	1.90
Mean value		2.40	2.70	3.10	-
LSD_{05}, cm: A = 1.56; B = 1.01; AB = 1.60					
Iturupskaya					
1/2 WPM	0.5	3.50±0.23	3.90±0.33	4.80±0.40	4.10
	1.0	2.00±0.17	2.20±0.18	2.50±0.20	2.20
1/4 WPM	0.5	2.00±0.26	2.50±0.31	2.90±0.34	2.50
	1.0	1.60±0.14	2.00±0.16	2.20±0.19	1.90
Mean value		2.30	2.60	3.10	-
LSD_{05}, cm: A = 1.62; B = 1.10; AB = 1.79					
Khabarovskaya					
1/2 WPM	0.5	3.60±0.30	4.10±0.36	4.60±0.39	4.10
	1.0	1.80±0.16	2.10±0.18	2.50±0.21	2.10
1/4 WPM	0.5	2.20±0.27	2.40±0.28	2.80±0.32	2.50
	1.0	1.60±0.14	1.90±0.15	2.40±0.20	1.90
Mean value		2.30	2.60	3.10	-
LSD_{05}, cm: A = 1.69; B = 1.18; AB = 2.01					

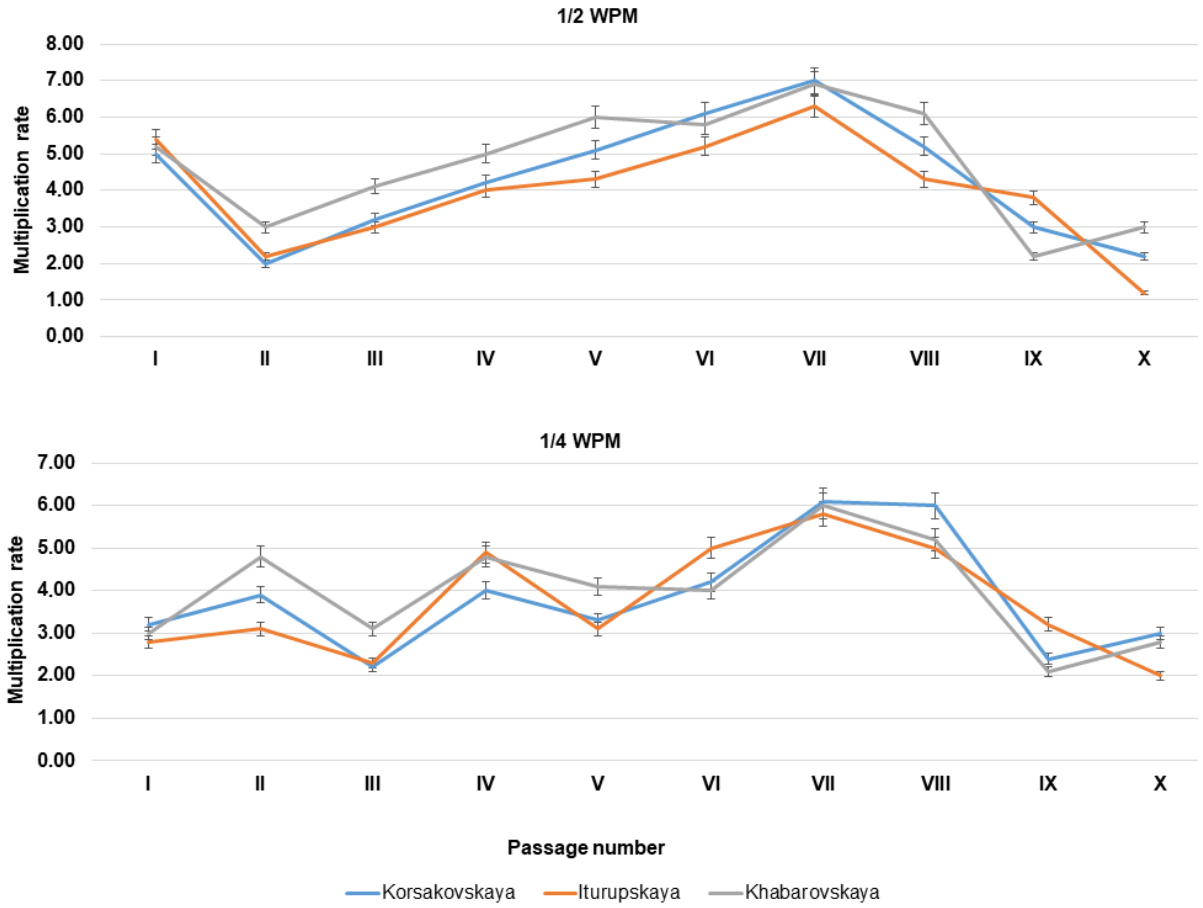

Fig. 6: Multiplication rate of *Vaccinium praestans* various forms in culture *in vitro* depending on the number of passages; A – 1/2 WPM; B – 1/4 WPM.

Table 5: ANOVA results for *Vaccinium praestans* shoot length in *in vitro culture* (n = 30; α = 0.05)

Source	SS	df	MS	F	p-value	F critical
Korsakovskaya						
Factor A	30.51	3	10.17	273.8779	1.09E-18	3.00878657
Factor B	3.165	2	1.5825	42.6167	1.27E-08	3.402826105
Factors A×B	0.255	6	0.0425	1.144524	0.367611	2.508188823
Inside	0.8912	24	0.037133			
Total	34.8212	35				
Iturupskaya						
Factor A	27.62568	3	9.208558	80.11506	1.21E-12	3.008787
Factor B	0.465	2	0.2325	2.022765	0.154231	3.402826
Factors A×B	11.555	6	1.925833	16.75488	1.62E-07	2.508189
Inside	2.7586	24	0.114942			
Total	42.40428	35				
Khabarovskaya						
Factor A	25.82	3	8.606667	255.7702	2.41E-18	3.008787
Factor B	3.635	2	1.8175	54.01189	1.3E-09	3.402826
Factors A×B	0.145	6	0.024167	0.718177	0.638801	2.508189
Inside	0.8076	24	0.03365			
Total	30.4076	35				

Table 6: The rooting per explant of *Vaccinium praestans* in *in vitro* cultures on 1/2 Woody Plant Medium culture medium, depending on the growth regulator concentration, %

Auxin additive (factor A)		Biostimulant additive (factor B)		
Auxin type	Concentration, mg L^{-1}	Without additives (control)	0.5mL L^{-1} Epin-Extra	0.1mL L^{-1} HB-101
IBA	1.0	80	88	89
	2.0	85	92	96
IAA	1.0	80	85	88
	2.0	88	90	93
Iturupskaya				
IBA	1.0	82	86	86
	2.0	84	90	91
IAA	1.0	80	90	88
	2.0	86	88	89
Khabarovskaya				
IBA	1.0	80	88	89
	2.0	85	90	94
IAA	1.0	81	87	90
	2.0	84	90	92

Depending on auxin and its concentration, there were no statistically significant differences in the number of *V. praestans* roots in *in vitro* culture at the stage of rhizogenesis. The number of roots in *V. praestans* plants when using both IAA and IBA varied on average from 1.8 to 2.3pcs. At a concentration of 2.0mg L^{-1}, which is by 20-54% more than at 1.0mg L^{-1}. There were no significant differences in the number of roots when adding growth-stimulating preparations Epin-Extra or HB-101 to the culture medium (Table 7). Callus on shoots was entirely absent in all treatments.

The reliability of the obtained data on the number of *V. praestans* shoots is confirmed by the results of the ANOVA analysis (F statistic value > F critical value; P<0.05) (Table 8).

The mean length roots of *V. praestans* in *in vitro* culture was longer when using IBA of 2.0mg L^{-1} and averaged 2.0-2.1 cm for all forms. The mean root length was by more than control by 100-120% in variant with adding 0.1mL L^{-1} of HB-101 was added to the medium and averaged 2.4cm, by 33-60% in variant with adding 0.5mL L^{-1} Epin-Extra and averaged 1.6-1.7 cm (Table 9).

The reliability of the obtained data on the *V. praestans* root length is confirmed by the results of the ANOVA analysis (F statistic value > F critical value; p-value < 0.05) (Table 10).

The data obtained are consistent with the results of our previous studies using the growth-regulating preparation HB-101 in clonal micropropagation of *V. praestans* at the stages of proliferation and rooting of micro-shoots. In some cases, the addition of 0.5mL L^{-1} of Epin-Extra was not inferior to the effectiveness of 0.1mL L^{-1} of HB-101, whereas when using 0.5mL L^{-1} of Zircon, the biometric indicators of *V. praestans* shoots and roots in *in vitro* culture were noticeably less (Chudetsky, 2022a).

Further, *V. praestans* plants rooted *in vitro* were transplanted into containers with peat substrates (Fig. 7). Later 10 days after transplanting *V. praestans* plants onto substrates without mulching, it was found that the adaptation of this species to non-sterile *ex vitro* conditions is most favorable in May.

Table 7: The number of *Vaccinium praestans* roots obtained in *in vitro* cultures on 1/2 Woody Plant Medium culture medium depending on the growth regulator concentration, pcs

Auxin additive (factor A)		Biostimulant additive (factor B)			Mean value
Auxin type	Concentration, mg L^{-1}	Without additives (control)	0.5mL L^{-1} Epin-Extra	0.1mL L^{-1} HB-101	
Korsakovskaya					
IBA	1.0	1.50±0.12	1.60±0.14	1.90±0.12	1.70
	2.0	1.80±0.15	2.30±0.20	2.20±0.18	2.10
IAA	1.0	1.10±0.09	1.50±0.12	1.80±0.14	1.50
	2.0	1.60±0.11	1.90±0.15	2.00±0.16	1.80
Mean value		1.50	1.80	2.00	-
LSD_{05}, pcs.: A = 1.17; B = 1.11; AB = 1.27					
Iturupskaya					
IBA	1.0	1.50±0.13	1.70±0.14	1.60±0.12	1.60
	2.0	1.90±0.15	2.00±0.17	2.40±0.20	2.10
IAA	1.0	1.50±0.11	1.40±0.10	1.50±0.11	1.50
	2.0	1.60±0.12	1.80±0.13	2.00±0.15	1.80
Mean value		1.60	1.70	1.90	-
LSD_{05}, pcs.: A = 1.30; B = 1.16; AB = 1.52					
Khabarovskaya					
IBA	1.0	1.60±0.12	1.80±0.13	1.40±0.10	1.60
	2.0	2.00±0.15	2.20±0.17	2.60±0.21	2.30
IAA	1.0	1.40±0.10	1.20±0.09	1.40±0.11	1.30
	2.0	1.60±0.11	2.00±0.16	2.40±0.20	2.00
Mean value		1.70	1.80	2.00	-
LSD_{05}, pcs.: A = 1.42; B = 1.21; AB = 1.73					

Table 8: ANOVA results for number of *Vaccinium praestans* shoots in *in vitro culture* (n = 30; α = 0.05)

Source	SS	df	MS	F	P-value	F critical
Korsakovskaya						
Factor A	1.94	3	0.646667	51.39073	1.35E-10	3.00878657
Factor B	1.415	2	0.7075	56.22517	8.77E-10	3.402826105
Factors A×B	0.265	6	0.044167	3.509934	0.012327	2.508188823
Inside	0.302	24	0.012583			
Total	3.922	35				
Iturupskaya						
Factor A	2.0475	3	0.6825	43.65672	7.1E-10	3.008787
Factor B	0.38	2	0.19	12.15352	0.000226	3.402826
Factors A×B	0.36	6	0.06	3.837953	0.007983	2.508189
Inside	0.3752	24	0.015633			
Total	3.1627	35				
Khabarovskaya						
Factor A	4.5488	3	1.516267	45.18675	5.01E-10	3.008787
Factor B	0.357017	2	0.178508	5.319785	0.012237	3.402826
Factors A×B	2.97905	6	0.496508	14.79661	5.07E-07	2.508189
Inside	0.805333	24	0.033556			
Total	8.6902	35				

Table 9: The mean length roots of *Vaccinium praestans* obtained in *in vitro* cultures on 1/2 Woody Plant Medium culture medium depending on the growth regulator concentration, cm

Auxin additive (factor A)		Biostimulant additive (factor B)			Mean value
Auxin type	Concentration, mg L^{-1}	Without additives. (control)	0.5mL L^{-1} Epin-Extra	0.1mL L^{-1} HB-101	
Korsakovskaya					
IBA	1.0	1.40±0.12	1.70±0.14	2.00±0.17	1.70
	2.0	1.20±0.10	1.60±0.13	3.40±0.30	2.00
IAA	1.0	1.10±0.09	1.60±0.12	2.00±0.18	1.60
	2.0	1.00±0.08	1.70±0.14	2.10±0.18	1.60
Mean value		1.20	1.70	2.40	-
LSD_{05}, cm: A = 1.27; B = 1.31; AB = 1.67					
Iturupskaya					
IBA	1.0	1.20±0.10	1.50±0.12	1.60±0.14	1.40
	2.0	0.90±0.08	1.80±0.15	3.80±0.32	2.10
IAA	1.0	1.30±0.10	1.70±0.15	2.30±0.19	1.80
	2.0	0.80±0.07	1.60±0.13	1.90±0.16	1.40
Mean value		1.00	1.60	2.40	-
LSD_{05}, cm: A = 1.45; B = 1.36; AB = 1.97					
Khabarovskaya					
IBA	1.0	1.30±0.12	1.40±0.12	1.80±0.16	1.50
	2.0	1.00±0.08	1.70±0.14	3.60±0.31	2.10
IAA	1.0	1.20±0.10	1.60±0.14	2.20±0.19	1.60
	2.0	0.90±0.08	1.70±0.15	2.00±0.18	1.50
Mean value		1.10	1.60	2.40	-
LSD_{05}, cm: A = 1.55; B = 1.42; AB = 2.19.					

Table 10: ANOVA results for *Vaccinium praestans* root length in *in vitro culture* (n = 30; α = 0.05)

Source	SS	df	MS	F	P-value	F critical
Korsakovskaya						
Factor A	1.42	3	0.473333	23.20261	2.84E-07	3.00878657
Factor B	8.765	2	4.3825	214.8284	4.81E-16	3.402826105
Factors A×B	3.095	6	0.515833	25.28595	2.93E-09	2.508188823
Inside	0.4896	24	0.0204			
Total	13.7696	35				
Iturupskaya						
Factor A	3.28	3	1.093333	68.15584	6.91E-12	3.008787
Factor B	10.98	2	5.49	342.2338	2.28E-18	3.402826
Factors A×B	5.96	6	0.993333	61.92208	2.02E-13	2.508189
Inside	0.385	24	0.016042			
Total	20.605	35				
Khabarovskaya						
Factor A	2.06	3	0.686667	64.67818	1.21E-11	3.008787
Factor B	10.32	2	5.16	486.0283	3.83E-20	3.402826
Factors A×B	4.42	6	0.736667	69.38776	5.64E-14	2.508189
Inside	0.2548	24	0.010617			
Total	17.0548	35				

Fig. 7: Adaptation of *Vaccinium praestans* 'Korsakovskaya' plants (height up to 2–3cm) rooted *in vitro* to *ex vitro* conditions on peat substrate later 14 days after transplanting (orig.).

The greatest survival rate was observed when using peat with sand 1:1 (91-93%) and peat with zeolite 1:1 (89-96%) when sprayed with water and treated with 0.1mL L^{-1} of HB-101, as well as on peat with vermiculite (1:4) and on top-type peat during treatment 0.1mL L^{-1} of HB-101 (90%) (Table 11). Also, the plantings were mulched with sphagnum moss, which has antiseptic properties, as well as contributing to moisture retention and increasing the lightness and breathability of the substrate. When mulching with sphagnum moss, the survival rate of adaptable *V. praestans* plants was slightly higher compared to the non-mulching variant, and the highest rates were also detected during transplantation in May in variants using peat + zeolite 1:1 (90-96%), peat with sand 1:1 (92-94%) when treated with 0.1mL L^{-1} HB-101 and spraying with water, and also on high-moor peat, on peat with vermiculite 3:1 and peat with perlite 3:1 when treated with 0.1mL L^{-1} HB-101 (90-94%).

At the same time, according to visual observations, in the variants of the experiment with sphagnum moss mulching, the development of contamination was not observed, whereas in the variants without mulching, contamination of single plants was noted (up to 10% of plants were contaminated and didn't survive due to infection by fungi).

V. praestans plants adapted to *ex vitro* conditions within 60 days were transplanted in open ground conditions on the same test plot in the conditions of the Kostroma region, Russia in the 3rd ten-day period of May (Fig. 8).

Fig. 8: *Vaccinium praestans* plants (height up to 4cm) obtained by the method of clonal micropropagation in open ground conditions of Kostroma region, Russia (Juny 2023) (orig.).

The survival rate of the plants obtained by the method of clonal micropropagation 14 days after transplantation was: Korsakovskaya form – 90%, Iturupskaya form – 86%, Khabarovskaya form – 81%. The survival rates of *V. praestans* plants on the 30th, 60th and 90th days after transplantation remained unchanged and also amounted to 81-90% depending on the form.

DISCUSSION

There are only a few studies on *in vitro* cultivation and *ex vitro* adaptation of regenerated *V. praestans* plants. In the study by Stanienė et al. (2002), it was noted that the highest shoot number (1.4-1.6pcs./shoot) of *V. praestans* and their length (15.9-16.9 cm) *in vitro* culture were observed on the WPM culture medium with the addition of 5mg L^{-1} of N6-(2-isopentenyl)adenine (2-iP). This is consistent with our experience using this nutrient medium, but our data showed higher shoot proliferation results when using 1/2 WPM and 1/4 WPM compositions with the addition of 0.5–1.0mg L^{-1} 6-BAP. Moreover, in the study of the same authors (Stanienė et al., 2002), the multiplication rate of *V. praestans* plants in *in vitro* culture was 2.9-3.3 shoots/explant, while in our case it reached from 4.0 to 7.0 shoots/explant. The choice of 6-BAP as a phytohormone in the nutrient medium in our study is due to the fact that it is a much more accessible and cost-effective cytokinin on the Russian market compared to 2-iP and zeatin, which also has a cytokinin nature. At the same time, 6-BAP is the most common and quite effective for shoot proliferation in clonal micropropagation of the different berry crops (Clapa et al., 2023; Valdivia-Rojas et al., 2025), including the genus *Vaccinium* (Cheremnykh et al., 2021; Makarov et al., 2021; Taku et al., 2025) and therefore its use is quite advisable.

According to the studies by Stanienė et al. (2002), the survival rate of regenerated *V. praestans* plants under *ex vitro* conditions on a sandy substrate was 33.3-100% depending on the form, while in our study, the maximum survival rates (88-91%) were noted when plants were transplanted in May on high-moor peat, peat:sand (1:1), and peat:zeolite (1:1). The positive experience of using the growth-regulating preparation HB-101 at the adaptation stage of *V. praestans* regenerants is consistent with the results of our other research on its use in the adaptation of *in vitro* obtained lingonberry (*Vaccinium vitis-idaea* L.) plants of the Kostromskaya Rozovaya, Kostromichka and Koralle cultivars to *ex vitro* conditions, where the survival rate of plants on high-moor peat and on peat:vermiculite (1:4) substrates was 93%, on peat:perlite (1:4) – 99%, on peat:sand (1:1) – 90% on peat substrates (Chudetsky et al., 2021). The highest survival rate of lowbush blueberry (*Vaccinium angustifolium* Ait.) microplants in the adaptation *ex vitro* was found on a peat + zeolite (3:1) substrate and amounted to 82-89% (Makarov et al., 2025). Kamchatka bilberry can grow on the rocky slopes, so volcanic zeolite as a component of the substrate has a fairly beneficial effect on the development of plants when adapting to non-sterile conditions.

Table 11: The percentage of viable *Vaccinium praestans* plants on the total number of tested plants in non-sterile *ex vitro* conditions depending on the time of transplant, substrate and treatment option later 10 days after transplanting, %

Transplant time (on average per month)	Substrate	The percentage of viable,%	
		Water (control)	0.1mL L^{-1} HB-101*
Without mulching			
March	High-moor peat sterile	50.20±2.96	48.40±0.66
	High-moor peat + river sand 1:1	57.00±1.14	52.20±0.48
	High-moor peat + vermiculite 3:1	46.20±3.70	56.10±1.43
	High-moor peat + perlite 3:1	42.00±0.64	47.30±2.13
	High-moor peat + zeolite 1:1	55.30±3.43	62.30±3.81
April	High-moor peat sterile	60.30±3.69	62.00±2.70
	High-moor peat + river sand 1:1	64.20±4.01	65.50±3.33
	High-moor peat + vermiculite 3:1	53.20±0.66	61.30±0.83
	High-moor peat + perlite 3:1	48.40±3.59	51.10±0.12
	High-moor peat + zeolite 1:1	60.10±0.97	74.20±2.65
May	High-moor peat sterile	88.00±5.26	90.10±7.17
	High-moor peat + river sand 1:1	91.10±2.12	93.20±0.69
	High-moor peat + vermiculite 3:1	76.20±4.14	90.30±0.43
	High-moor peat + perlite 3:1	68.00±1.66	88.20±4.72
	High-moor peat + zeolite 1:1	89.40±0.41	96.00±3.44
Mulching with sphagnum moss			
March	High-moor peat sterile	54.20±0.62	52.10±1.52
	High-moor peat + river sand 1:1	48.00±0.59	46.40±1.97
	High-moor peat + vermiculite 3:1	39.30±2.30	52.20±0.17
	High-moor peat + perlite 3:1	56.20±0.44	48.10±3.77
	High-moor peat + zeolite 1:1	46.00±3.20	50.20±2.52
April	High-moor peat sterile	44.20±2.83	48.00±3.02
	High-moor peat + river sand 1:1	50.20±1.46	36.20±0.59
	High-moor peat + vermiculite 3:1	54.00±1.55	46.20±2.12
	High-moor peat + perlite 3:1	42.20±2.35	56.00±0.47
	High-moor peat + zeolite 1:1	54.50±2.82	62.20±0.06
May	High-moor peat sterile	90.00±2.31	93.30±2.91
	High-moor peat + river sand 1:1	94.20±5.98	92.10±2.12
	High-moor peat + vermiculite 3:1	72.50±2.51	90.20±0.21
	High-moor peat + perlite 3:1	92.10±2.02	94.00±1.68
	High-moor peat + zeolite 1:1	90.50±2.35	96.10±4.71

* Watering with a solution of the growth-stimulating preparation HB-101 at a concentration of 0.1mL L^{-1}

Our data are consistent with the other experiences on *in vitro* cultures of berry plants of genus *Vaccinium* (blueberries, cranberries, lingonberries) and confirm the positive results of using the same compositions of nutrient media and growth regulating preparations (Epin-Extra, HB 101) (Makarov et al., 2021; Chudetsky et al., 2022a) as well as the use of *Sphagnum* L. moss for adaptation of regenerated plants to non-sterile conditions *ex vitro* (Bozhiday & Kukharchik, 2014; Bozhiday, 2018; Chudetsky et al., 2022b). Our results confirm the prospects of their use as important factors for improving technology of accelerated propagation of forest berry plants. The obtained results can be used to improve the elements of the technological cycle of accelerated cultivation of *V. praestans* using the clonal micropropagation method.

Considering the successful results obtained in *ex vitro* conditions, in the future, it would be advisable to conduct research using temporary immersion bioreactor systems (bioreactors) to scale up *V. praestans* micro-plants for industrial purposes.

Conclusion

In summary, with clonal micropropagation of *V. praestans*, the number of micro-shoots of plants and their length at the stage of proliferation were significantly greater in variants with the 1/2 WPM culture medium, while the maximum values of the length of micro-shoots were achieved with the addition of 1.0mg L^{-1} 6-BAP together with 0.1 mL L^{-1} HB-101. The length of *V. praestans* roots in *in vitro* culture at the stage of rhizogenesis was the greatest on the 1/2 WPM culture medium with simultaneous addition of 2.0mg L^{-1} IBA and 0.1mL L^{-1} HB-101. When adapting to non-sterile *ex vitro* conditions, the highest survival rate (90% and higher) of regenerating plants is *V. praestans* was observed during transplantation in May on peat substrates with the addition of zeolite, river sand and perlite, which is apparently due to increased aeration and water-carrying capacity of the soil when these components are added to sterile high-moor peat. At the same time, the use of a mulching layer of sphagnum moss prevented the development of contamination in plantings and contributed to an increase in plant survival rates. The obtained data indicate the prospects for growing *V. praestans* plants obtained by clonal micropropagation in the natural and climatic conditions of the Kostroma region, Russia. The method of clonal micropropagation is advisable to multiplicate *V. praestans* planting material for further cultivation on an industrial scale, and our results can be used for improving and optimizing the technology.

For obtaining *V. praestans* planting material *in vitro*, it is recommended to: initiate with sterilization of explants from apical meristems in 0.2% $AgNO_3$ solution for 10min; proliferate on 1/2 WPM with 1.0mg L^{-1} 6-BAP + 0.1mL L^{-1}HB-101; root on 1/2 WPM with 2.0mg L^{-1} IBA + 0.1 mL L^{-1} HB-101; harden in May on peat:sand (1:1) or peat:zeolite (1:1), or peat:perlite (3:1), optionally mulch with sphagnum moss.

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