IRSTI 634.1/.7; 602.6:59; 602.6:612; 578.2:21; 631.589.2 https://doi.org/10.26577/IJBCh20251818

A.B. Tolegen[1,2], N.V. Romadanova[1*], M.M. Aralbayeva[1], N.K. Rymkhanova[1,2], Y.V. Ilkhatova[3], M.M. Agahanov[3], N.V. Mikhailenko[1], S.V. Kushnarenko[1]

[1]Institute of Plant Biology and Biotechnology, Almaty, Kazakhstan
[2]Al-Farabi Kazakh National University, Almaty, Kazakhstan
[3]N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR), Saint Petersburg, Russia
*e-mail: nataromadanova@gmail.com
(Received 12 May 2024; received in revised form 26 June 2025; accepted 27 June 2025)

Utilizing Plant Preservative Mixture™ to eliminate endophytic bacterial contamination and establish *in vitro* cultures of blackberry varieties

Abstract: Bacterial, fungal, and viral contaminations pose a pervasive challenge to *in vitro* propagation of berry crops, presenting significant economic hurdles for the preservation of plant genetic resources and the commercialization of micropropagation. This study aimed to diagnose endophytic and viral contamination in commercial blackberry cultivars and while developing an *in vitro* method to eliminate bacterial contamination from the Chacanska Bestrna variety. The Plant Preservative Mixture™ (PPM™) was utilized as a broad-spectrum bactericidal agent within the blackberry *in vitro* tissue culture. Contaminated shoots were cultured on Murashige and Skoog medium supplemented with 0.2% v/v PPM™, maintained over a 12-week period through three subcultures, before being transferred to medium devoid of PPM™ for an additional 12 weeks. The results were evaluated after 12 and 32 weeks of culture on medium without PPM™. This study demonstrated that PPM™ is an effective control agent and can be successfully used to manage the growth of endophytic contamination. For the Chacanska Bestrna blackberry variety, 100% aseptic shoots were obtained *in vitro* without any negative effects on the shoot growth. However, the results indicate the opportunity for further testing of PPM™ on other blackberry varieties experiencing bacterial contamination, as it is possible that the contamination in this study was superficial and the selected concentration of the bactericide proved to be successful. To obtain virus-free planting stocks, *in vitro* rooted shoots were transferred to a soil substrate consisting of peat (40%), black soil (50%), and perlite (10%), and then adapted in a greenhouse at 20-23°C. The survival rate of blackberry varieties in greenhouse conditions was 93-99%.
Key words: blackberry; tissue culture; *in vitro* contamination; micropropagation; broad-spectrum biocide.

Introduction

The cultivation of berries stands as one of the most vibrant and flourishing sectors within the realm of agriculture, marked by its dynamic growth and lucrative potential. According to the Food and Agriculture Organization (FAO), the Republic currently produces about 420 thousand tons of fruits and berries, while the population of the country requires more than 2.5 million tons of products according to world standards; about 70% of fruit and berry products are imported [1]. One of the limiting factors in the development of this industry in Kazakhstan is the lack of high-quality planting material. In this regard, the development of micropropagation techniques to obtain healthy planting material for berry crops is extremely important.

Blackberry is a valuable berry crop, the fruits of which have high nutritional and healing properties, due to their rich chemical composition. These exquisite berries possess approximately 10% carbohydrates along with a medley of organic acids, pectin, fiber, and a rich array of vitamins including P, B, E, and C, complemented by a diverse spectrum of macro and microelements [2]. The allure of blackberries extends beyond their delightful taste and captivating aroma; they are celebrated for their robust concentrations of anthocyanins and phenols, both of which

exhibit powerful antioxidant and anticancer properties [3]. Furthermore, blackberries lend themselves to the creation of high-quality processed delicacies, such as jams, jellies, marmalade, juices, and extracts, as well as delightful wines and beverages. These fruits are equally adept at swift freezing, perfect for crafting vibrant food colorings that enhance culinary creations. Despite the high value of this berry crop, blackberries are still not as popular among farmers in Kazakhstan as raspberries and strawberries. The main difficulties in cultivating this crop are associated, first of all, with the need to select high-quality, highly productive varieties suitable for the soil and climatic conditions of the country, as well as the use of modern agricultural technologies [4]. In addition, Kazakhstani farmers use blackberry planting stocks purchased from foreign producers because there is no high-quality, pathogen-free material of domestic production available.

Traditionally, varieties of berry crops are propagated by green or woody cuttings, which is often not very effective [5-7]. Micropropagation is currently the only way to produce healthy planting material on an industrial scale, widely used in many countries [8]. The development of blackberry micropropagation technologies is being fruitfully pursued in the USA [9], Russia [10-11], and European Union countries [12-13]. There are no scientific publications on micropropagation of blackberries in Kazakhstan.

When producing planting material, it is very important to monitor its virological status. Blackberry varieties are propagated vegetatively and can accumulate viruses during various development stages. More than 30 different viruses are known to infect native and cultivated blackberries [14-16]. *Raspberry bushy dwarf virus* (RBDV) is the most harmful and widespread pathogen of *Rubus* genus including blackberry [17]. RBDV was discovered worldwide in Rubus commercial growing regions, including North and South America, Europe, China, and New Zealand [13-19, 17-23]. The occurrence of RBDV both in cultivated and native raspberry plants was recently reported in Kazakhstan [24]. *Blackberry yellow vein associated virus* (BYVaV) has been described in the USA in blackberries with symptoms of vein clearing, yellow mottling and plant decline [25]. Black raspberry (*Rubus occidentalis*) plantings in Oregon (USA) also declined by *Black raspberry necrosis virus* (BRNV) [26]. *Apple mosaic virus* (ApMV), which is widespread throughout the world, has also been found in symptomless Rubus plants in the United States and Germany [16].

One of the indispensable stages of micropropagation is *in vitro* culture initiation. Contamination of field plant material with bacterial and fungal pathogens can significantly reduce the percentage of aseptic shoots. Surface disinfection helps eliminate epiphytic bacterial and fungal contaminations; however, sterilizing agents usually have no effect on endophytic bacteria that inhabit inner tissues and organs [8, 27, 28, 29]. A wide variety of endophytic bacteria have been identified in tissue culture of various plants, including Curtobacterium, Mycobacterium, Paenibacillus, Pseudomonas, Stenotrophomonas found in *in vitro* shoots of *Rubus ideaus* [25, 30]. Antibiotic treatment has been shown to be effective in eliminating bacterial contamination from micropropagated strawberry plants [31]. A combination of several antibiotics may have advantages over the use of a single antibiotic in preventing the emergence of resistant strains [27, 31, 32]. The use of the broad-spectrum biocide Plant Preservative MixtureTM (PPM^{TM}) has shown high efficiency in eliminating endophytic contamination from explants taken in field collections of various plants and propagated *in vitro*: rhododendron and European birch [33], *Acacia* [32], *Malus* [35], and walnut [36].

The purpose of this work was to study the weight of PPM^{TM} on the elimination of endophytic bacteria and the production of blackberry varieties aseptic plants.

Materials and methods

Plant Material. One-year-old shoots of three blackberries varieties were cut for *in vitro* initiation in June 2023 from the field collection of the "Semirechye" nursery in Almaty region. Three blackberry varieties were used: Chester, Natchez (USA) and Chacanska Bestrna (Serbia).

In vitro Culture Initiation and Micropropagation. Cuttings approximately 15-20 cm long were washed in soapy water and rinsed in tap water, then apices (1.5-2.5 cm long) were excised from the stem and in a laminar flow hood were surface disinfected with 0.1% mercuric chloride with a few drops of "Tween 20" for 5 or 7 min. Following washing with sterile water, the delicate shoot apices were placed within 25 × 250 mm glass tubes, cradled in Murashige and Skoog medium (MS) [35] enriched with doubled dose of iron chelate, 0.5 mg L^{-1} 6-benzylaminopurine (BAP), 0.01 mg·L^{-1} indolyl-3-butyric acid (IBA), 30 g·L^{-1} sucrose, 4 g·L^{-1} agar sourced from (PhytoTechnology Laboratories®, Lenexa, KS, USA), 1.25 g·L^{-1} GelriteTM (PhytoTechnology Laboratories®,

Lenexa, KS, USA). The medium was adjusted to pH 5.7 using 0.1 N NaOH, followed by autoclaving at 121°C for 20 min.

The burgeoning shoot cultures thrived within a growth room-maintained 24°C basking under a 16-hour photoperiod (light intensity of 40 μmol·m^{-2}·s^{-1}) illuminated by the gentle glow of two types of OPPLE tubular fluorescent lamps: YK21RR 16/G 21 W 6500 K RGB and YK21RL 16/G 21 W 4000 K RGB (Opple, China).

The number of viable shoots, shoots with bacterial and fungal contamination, and necrosis were evaluated using visual inspection during the first two weeks culturing. Visually clean shoots were indexed for endophytic contamination using 523 detection medium [36]. The bases of the shoots were placed in Petri dishes with 523 medium with 10 g·L^{-1} sucrose, 8 g·L^{-1} casein hydrolysate (Sigma-Aldrich, St. Louis, MO, USA), 4 g·L^{-1} yeast extract, 6 g·L^{-1} GelriteTM, 2 g·L^{-1} KH_2PO_4, 0.15 g·L^{-1} $MgSO_4 \cdot 7H_2O$, pH 6.9.

Contaminated shoots 2-3 cm in size with 3-5 leaves, after testing on the 523 medium with identified endophytic bacterial contamination were divided into 2 parts: 1) cultivated on MS medium mentioned earlier for three passages during 4 weeks each – Control; 2) cultured on MS medium with 0.2% (volume/volume,) for three passages during 4 weeks each (Experiment 1 (E1). After this all shoots were transferred to MS medium without PPM$^{TM.}$ for three subcultures during 4 weeks each (Experiment 2 (E2). After each cultivation, indexing for endophytes was carried out using 523 medium. After experiment E2, *in vitro* shoots were further propagated under the conditions described above, after 8 cultivations of 4 weeks each (32 weeks), *in vitro* shoots were again tested on 523 medium (Experiment 3 (E3).

Virus detection. In vitro shoots were evaluated for five viruses: *Raspberry bushy dwarf virus* (RBDV), *Strawberry necrotic shock virus* (SNSV), *Apple mosaic virus* (ApMV), *Black raspberry necrosis virus* (BRNV), and *Blackberry yellow vein associated virus* (BYVaV) by multiplex reverse transcription polymerase chain reaction (RT-PCR). Sequences of oligonucleotides used for virus detection are presented in Table 1.

Total RNA was extracted from *in vitro* blackberry leaves using the CTAB method [39, 40, 41]. The quality of the exported RNA was evaluated in Tris-acetate buffer (TAE) by electrophoresis in 2% agarose gel. Reverse transcription was carried out in a reaction mixture with a volume of 20 μl, which contained 0.5 μg/μl of universal primer and 200 units of reverse transcriptase RevertAid. At the first stage, a mixture containing total RNA (at least 200 ng) and a universal primer was incubated at 72°C for 10 min. Next (second step), to a 15 μL reaction mixture 4 μL of 5x Buffer RT, 200 μM dNTP, and RevertAid reverse transcriptase were supplemented. cDNA synthesis at 45°C for 60 min was carried out. The reaction mixture for PCR contained 2 μl of cDNA, 1x Dream Taq Buffer, 200 μM dNTP, 0.2 μM reverse and forward specific primer for each virus and 1.5 units Dream Taq polymerase (Thermo Fisher Scientific Inc., Vilnius, Lithuania).

Next, MasterMixt was prepared using the following components: 8.80 μl of H_2O, 1.80 μl of 10X reaction mixture, 0.45 μl of 100 mM $MgCl_2$. 1.20 μl 5 mM dNTPs, 1.80 μl primer F, 1.80 μl primer R, 0.15 μl Taq polymerase. The reaction program consisted of the following stages: denaturation at 95°C for 5 min; 30 cycles: denaturation for 30 sec at 95°C, aniling for 20 sec at 52°C, elongation for 40 sec at 72°C; last elongation at 72°C for 5 min. Amplification products in a 2% agarose gel in Tris-borate buffer (TBE) were investigated by gel electrophoresis. Confirmation of the RNA purity was obtained by the separation of the 28S and 18S ribosomal RNA on the electropherogram (Thermo Fisher Scientific Inc., Vilnius, Lithuania) [43].

Obtaining of Virus-Free Blackberry Planting Stocks. To obtain planting stocks, rooted *in vitro* plants were transferred to (250 ml) polyethylene containers with soil substrate: black soil ("Ahty" https://almaty.flagma.kz/342682/), peat (Kekkila DSM 1 W EXTRA, Finland), and perlite (Union Perlite, Almaty, RK https://perlite.kz/) in a percentage correlation of 50:40:10. The containers with the plants were covered with transparent plastic caps and kept to a three-layer polyethylene film greenhouse (240 m²) with a temperature range from 18°C to 25°C and humidity from 40% to 80%. The temperature regime in the greenhouse was controlled using a floor-standing column-type air conditioner, Almacom ACP-60A (Almacom, China). The influence of temperature and humidity in the greenhouse on the survival and adaptation of the plants was monitored. After one week, the plastic caps were taken out for a period – 10-15 min, and later on, the ventilation duration was increased to 8 h. The adaptation period was for 3-6 weeks. For continued adaptation, the plastic caps were taken out.

Table 1 – Sequences of oligonucleotides used for virus detection.

Virus	Primer name	Sequence (5'→3')	Amplicon size (bp)	Annealing temperature (°C)	Reference
ApMV	*ApMV-F*	AGGGTCCTGAGCAGTCGAGA	264	64	[16]
	ApMV-R	GTTTGGAGGGGCTTCCCACT			
BRNV	*BRNV-F*	ATGCTGAGCCACTTGTGA	417	54	[24]
	BRNV-R	ATCTGGTGTGTTCCGCAT			
BYVaV	*BYVaV-F*	CGTAAGAAGTTCAACATCCA	180	58	[25]
	BYVaV-R	CTTCCAGAATAGAGCACTCG			
RBDV	*RBDV-F*	TTCATCCTCCAAATCTCAGCAAC	245	73	[26]
	RBDV-R	CGTCGACGGCACCGCCCACCACA			
SNSV	*SNSV-F*	CAGTGTTTACGGCTGCGAAG	823	69	[42]
	SNSV-R	GGGATCGATTGGTTAGGACCGTCAT			

Statistical Analysis. Fifteen explants of each variety for each treatment in triplicate (n=45) were used to initiate *in vitro* culture. For testing on the 523 medium, *in vitro* shoots were used in triplicate (n = 15). Results were statistically processed as the mean values ± standard error (SE). Means were compared using SYSTAT 13.0, Statistics Software; SYSTAT Software, Inc.: San Jose, CA, USA, 2009 [44]

Results and discussion

In Vitro Culture Initiation. After placing the surface-disinfected apexes into tubes containing culture medium (Figure 1), a visual inspection was conducted after one week to assess the number of viable explants, as well as the occurrence of necrosis and contamination. The results of visual inspection of the *in vitro* apexes of the three varieties Chester, Natchez and Chacanska Bestrna are presented in Figure 2.

Fungal contamination was not detected in any variety and any disinfection duration. Epiphytic bacterial contamination was noted in a small number of explants: with 5 minutes of disinfection – from 2.2 to 6.7%, on average 5.2%; with 7 minutes of disinfection – from 0 to 4.4%, on average 2.2%. The percentage of necrosis significantly increased in the apices of the Chester and Chacanska Bestrna varieties when the treatment duration increased to 7 minutes and averaged 47.4% for the three varieties compared to 24.4% when treated for 5 minutes. The percentage of viable apices without visual signs of contamination was significantly higher with a 5-minute treatment in the Chester and Chachanska Bestrna varieties and on average for the three varieties was 70.4% compared to 50.4% with a 7-minute treatment.

In general, disinfection with mercuric chloride for 5 minutes was a gentler treatment, but sufficient to eliminate epiphytic contamination and obtain 60.0% viable shoots for 'Chacanska Bestrna', 71.1% for 'Chester' and 80.0% for 'Natchez' (Figure 2).

In contrast, all shoots of Chacanska Bestrna were affected by endophytic contamination, as shown in Figure 4 c highlighting the importance for further study the reasons it's susceptibility to endophytes. The results of testing on medium 523 can serve as the basis for optimizing protocols for different varieties, which in turn will increase the overall productivity and stability of the crop.

Virus detection. *In vitro* shoots of all three blackberry varieties were tested for the presence of five viruses: *Raspberry bushy dwarf virus* (RBDV), *Strawberry necrotic shock virus* (SNSV), *Apple mosaic virus* (ApMV), *Black raspberry necrosis virus* (BRNV), and *Blackberry yellow vein associated virus* (BYVaV). This test is necessary to assess virus infection and ensure the quality of cultures. Identifying viruses in the early stages allows to take measures to eliminate them and minimize risks during further plant propagation. Virus load research researches allow to select the most resistant varieties for successful micropropagation and improved agronomic characteristics. As a result, viruses were absent in all samples as shown on Figure 4.

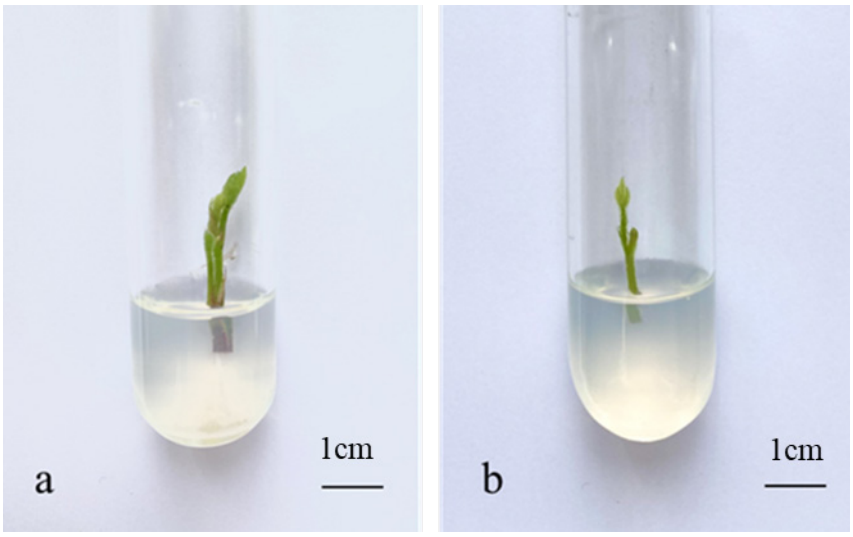

Figure 1 – Shoot apexes of blackberry cultivars Natchez (a) and Chacanska Bestrna (b) newly cultured on Murashige and Skoog medium with doubled dose of iron chelate, 0.5 mg·L^{-1} 6-benzylaminopurine, 0.01 mg·L^{-1} indolyl-3-butyric acid, 30 g·L^{-1} sucrose, 4 g·L^{-1} agar, 1.25 g·L^{-1} Gelrite^{TM}, pH 5.7. Scale bars = 1 cm

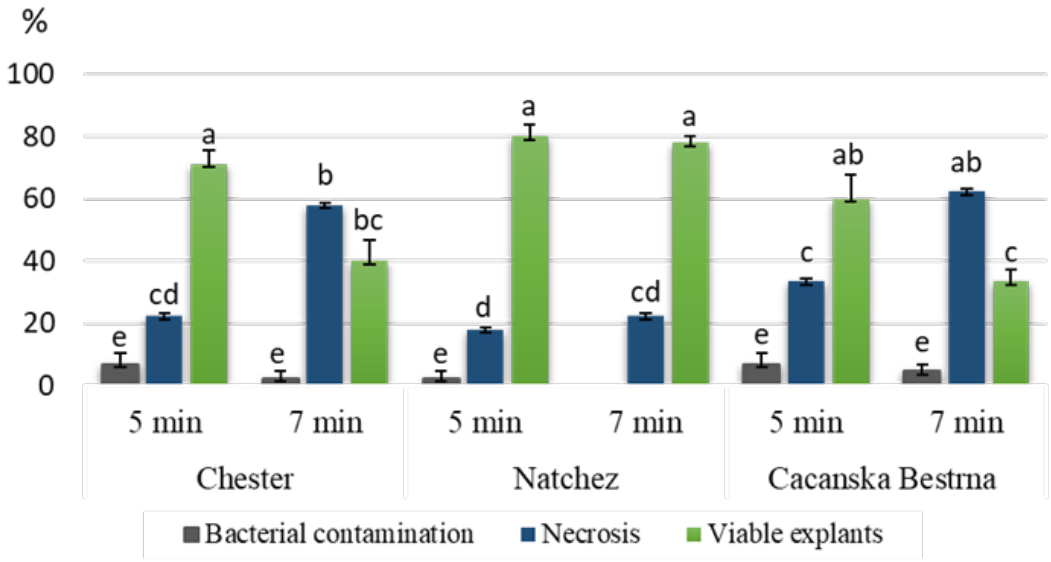

Figure 2 – Effect of duration disinfection with $HgCl_2$ (5 or 7 min) during *in vitro* initiation three blackberry varieties Chester, Natchez, and Chacanska Bestrna. The results are average values ± standard error (SE). Means denoted by different letters are significantly different at p≤ 0.05 using Tukey's mean separation test

Detection of Endophytic Contamination. Contamination of plant tissue culture by pathogens is indeed a serious problem in micropropagation, because even apparently aseptic shoots can contain endophytes contamination [44-45]. Careful inspection of explant purity is important to minimize the risk of endophytic contamination. The use of a specialized 523 medium allows to detect of these contaminants, improves the quality of the culture and increases the success of micropropagation and further cryopreservation of shoot tips [28, 36, 45]. The study revealed that all shoots of the Natchez and Chester varieties were aseptic, which may indicate their high resistance to infections, as shown in Figure 3.

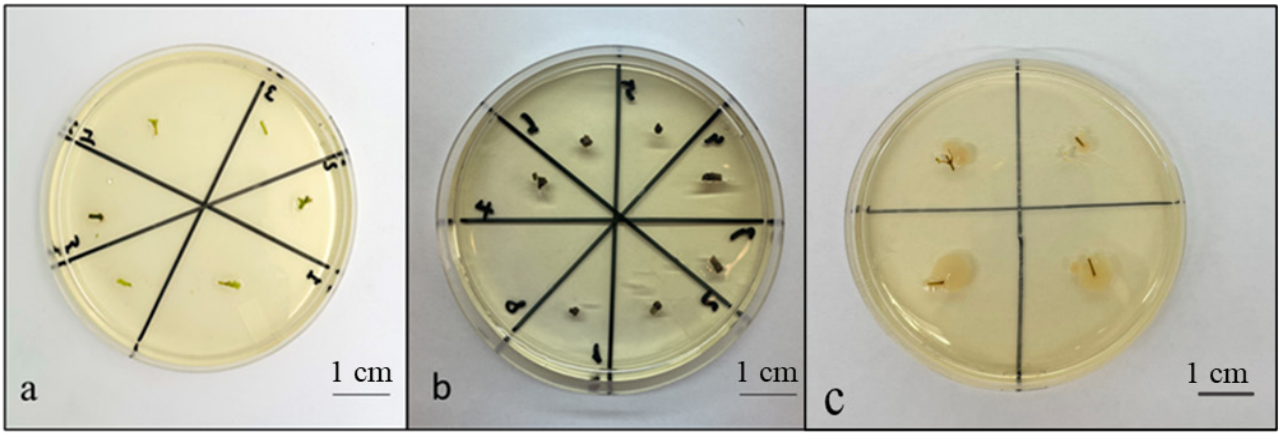

Figure 3 – Testing *in vitro* blackberry shoots for contamination on specialized bacteriological growth medium 523. Clean cultures of Chester variety (a) and Natchez variety (b). Bacterial growth on and around shoot initials of the Chacanska Bestrna variety shoots on specialized bacteriological growth medium 523 (c). Scale bars = 1 cm

The absence of virus contamination may be attributed to the fact that the source material was obtained from certified planting stocks grown in container culture. Since these planting stocks were not propagated in open ground, they were less exposed to potential virus contamination, ensuring a virus-free plant material. This finding indicates that the *in vitro* cultures of all tested blackberry varieties are free from virus contamination, this is essential for future micropropagation, cryopreservation and planting stocks obtaining.

Obtaining contamination-free in vitro shoots using chemotherapy with PPM™. In light of the pervasive endophytic contamination affecting all specimens of the Chacanska Bestrna variety, subsequent experiments called for the transplantation of *in vitro* shoots from this accession into a basic MS medium, enhanced with the bactericide PPM™. Following a meticulous examination for the absence of endophytic microflora on the specialized bacteriological medium 523, it was established that the formation of colonies on the MS medium with PPM™ had ceased entirely. The medium itself transformed, devoid of any signs of contamination. Thus, the addition of PPM™. to the culture medium gave good results, demonstrating the cessation of microbial growth as shown on figure 4. As a result, a method was developed for obtaining blackberry *in vitro* shoots that are free from endophytic contamination. The results of the set free of *in vitro* shoots from contaminant infection are given in the Table 2.

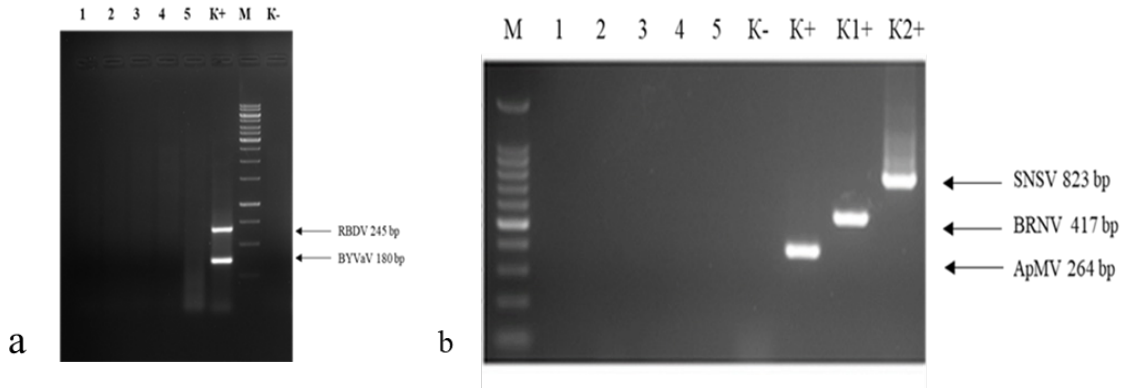

Figure 4 – Representative electrophoretic analysis of RT-PCR products from micropropagated blackberry accessions to test for the presence of five viruses: *Raspberry bushy dwarf virus* (RBDV), *Strawberry necrotic shock virus* (SNSV), *Apple mosaic virus* (ApMV), *Black raspberry necrosis virus* (BRNV), and *Blackberry yellow vein associated virus* (BYVaV). (a) K+ RBDV and BYVaV. (b) K+ SNSV, BRNV and ApMV. M – DNA marker for electrophoresis 100bp Plus (ThermoFisher), K – negative control; 1-2 – Chester, 3-4 – Natchez, 5 – Chacanska Bestrna

Specialized medium 523 contains amino acids from hydrolyzed casein, vitamins from yeast extract and sucrose as an energy source, therefore it is an ideal medium for bacteria and fungi to grow. In current experiments, contamination was observed in 100% of the control shoots (without PPM™ treatment) after 4 weeks of cultivation on specialized medium 523, contamination was already evident at the first week, indicating the effectiveness of this specialized bacteriological medium in detecting contamination. Therefore, our results show that PPM™ controlled *in vitro* shoots contamination on medium with PPM™ (0.2% v/v) for three cycles with 4 weeks duration (E1), the aseptic rates of shoots ranged from 46.7% to 100% (Table 2, E1) (Figure 5).

Results represent mean ± standard error (SE). Control comprised of shoot basal segment were isolated from four-week-old cultures with contamination and cultured on basal medium without PPM™ for three passages of four weeks each (total of 12 weeks of cultivation). Average values denoted by different letters within each section were significantly different at $p \leq 0.05$ using Tukey's mean separation test.

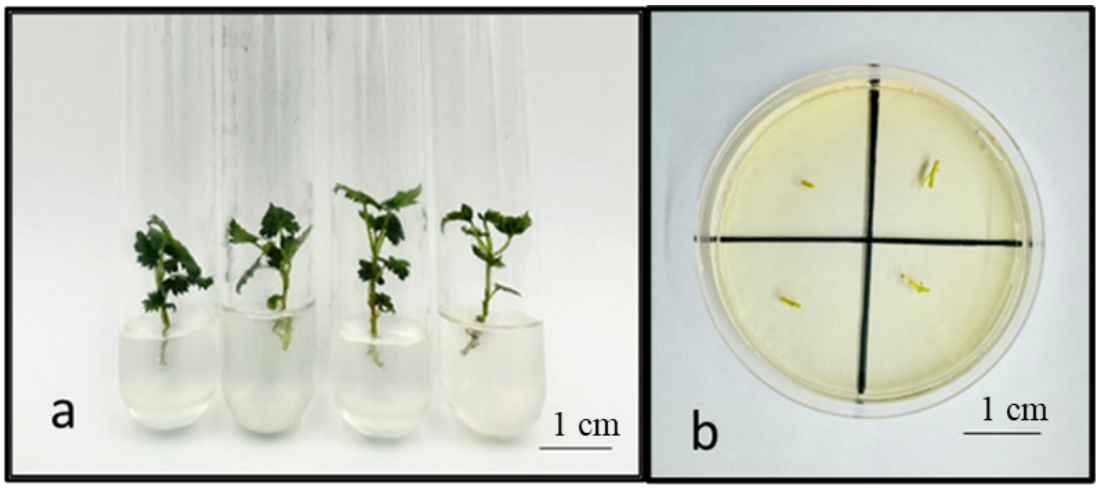

Figure 5 – Chacanska Bestrna variety four weeks after subcultivation on MS with doubled dose of iron chelate, 0.5 mg·L^{-1} BAP, 0.01 mg·L^{-1} IBA, 30 g·L^{-1} sucrose, 4 g·L^{-1} agar, 1.25 g·L^{-1} Gelrite™, 0.2% (v/v) PPM™, pH 5.7. (a) Testing Chacanska Bestrna *in vitro* shoots for the contamination on specialized bacteriological growth medium 523. Shoot were grown 12 weeks (three subcultures of 4 weeks each) on MS medium with PPM™ (0.2% v/v) and then were transferred to MS medium without PPM™ and cultured for three additional subcultures (total of 24 weeks of cultivation). No bacterial growth detected (b). Scale bars = 1 cm

Table 2 – Percentage of aseptic blackberry Chacanska Bestrna *in vitro* shoots cultures grown for 12 weeks (three passages by 4 weeks duration each) in a medium with 0.2% v/v Plant Preservative Mixture™ (PPM™) (Experiment 1, E1), later pre-treated shoots from E1 grown in a medium without PPM™ for 12 weeks (three passages by 4 weeks duration each) (Experiment 2, E2) and then the 8 cultivation by 4 weeks duration each (32 weeks) from E2 (Experiment 3 (E3)

Experiment name	Number of subcultures	[z]Aseptic shoots (%)
Control	the first subculture	0[a]
	the second subculture	0[a]
	the third subculture	0[a]
Experiment 1 (E1)	the first subculture	46.7±6.7[b]
	the second subculture	100[d]
	the third subculture	100[d]

Continuation of the table

Experiment name	Number of subcultures	[z]Aseptic shoots (%)
Experiment 2 (E2)	the first subculture	86.3±17.4[c]
	the second subculture	96.6±3.9[d]
	the third subculture	100[d]
Experiment 3 (E3)	the eighth subculture	100[d]

Shoots obtained from E1 were transferred to medium without PPM™. and cultured for more than three subculture cycles by 4 weeks duration each (12 weeks in total) before testing microbial growth (E2). After 12 weeks of cultivation, bacterial contamination was completely removed. Further testing on 523 medium of *in vitro* shoots, cultured on MS medium without PPM™ after 28 weeks (Experiment 3 (E3) also showed the absence of contaminated plants.

Studies by many authors show positive dynamics of using PPM™ for rejection contamination *in vitro* shoot culture. As the results show, the effect of PPM™ varies for different genotypes from 0 to 100% of aseptic shoots, which may indicate that the efficiency of that procedure for elimination of endophytic contamination condition on the type of tested plants and, mainly, on the type of contamination that has multiplied in the plant tissue [48-53.]. In the study obtaining, 100% of aseptic shoots were obtained *in vitro* for the blackberry variety Chacanska Bestrna without any negative effect on the growth of cultures *in vitro*. Perhaps this is due to the blackberry culture itself, since two of the three studied varieties were not at all susceptible to the action of contamination; perhaps the contamination was superficial and the selected concentration of the bactericide was successful.

Obtaining of Virus-Free Blackberry Planting Stocks. Already now in many countries it has significantly displaced raspberries, as it significantly surpasses them in terms of yield, transportability and medicinal properties. Commercially valuable varieties, such as those mentioned in this study, are unique thornless breeding specimens adapted to cultivation conditions. With proper agricultural techniques, their yield can reach 16-28 tons per hectare. If we consider that the original planting stocks are virus-free and free from endophytic contamination, the yield can be increased to 35 tons per hectare [1].

To obtain the planting stocks, shoots with roots were taken from a virus-free *in vitro* collection, free from endophytic contamination. It should be noted that blackberry root *in vitro* formation occurs independently on MS medium for micropropagation.

Rooted blackberry plants were transplanted into a soil substrate consisting of a mixture of black soil, peat, and perlite. For a week, the plants were adapted to reduced humidity by removing the plastic caps from the cultivation containers.

To ventilate the plants, the plastic cap was removed for 10-15 min, and in the next few days, the ventilation duration was increased to 8 h. The duration of planting stocks adaptation amounts from 3 to 6 weeks. In the greenhouse, where the plants were adapted, temperature and humidity were monitored daily. The survival rate of blackberry plants in the greenhouse at 20-23° reached 99.0%, while a decrease or increase in temperature led to a slight decrease in the survival rate of planting stocks to 94.3% and 93.0% respectively. When comparing statistical differences, no difference was noted between the options. However, when averaging the data, it was found that the optimal temperature for transferring aseptic plants to the soil is 20-23°C. Visually, the plants were also indistinguishable. Planting stocks adapted to greenhouse conditions are shown in Figure 6.

The planting stocks obtained from *in vitro* culture, of a high purity category and adapted to field conditions, will be used for the establishment of an elite nursery, as well as for mass production for subsequent commercialization within the framework of the project. Besides, the *in vitro* accessions will serve for conducting a wide range of biological research, including the long preservation and genetic resources international exchange for the creation of duplicate collections.

Figure 6 – Planting stocks of blackberry cultivar Chacanska Bestrna transplanted into a soil substrate: black soil, peat, and perlite (50:40:10) at 20–23°C and humidity from 40% to 80%. Scale bars = 1 cm

Conclusion

Bacteria, fungi and viruses are the constant threat to growth *in vitro* plants, however methods for controlling and, in some cases, eliminating contamination are already developed. In this research, the using of PPM™ (0.2% v/v) for 12 weeks of cultivation was found to be effective in controlling endophyte growth *in vitro* tissue cultures of the Chacanska Bestrna blackberry variety. Given that only one variety was affected by contamination in this case, there is a need for further testing of PPM™ on other blackberry varieties with bacterial outbreaks to confirm the study's results or for further investigation. From virus-free *in vitro* shoots of three blackberry varieties free from endophytic contamination, planting stocks adapted to greenhouse conditions (20-23°C) and soil substrate were obtained, that suitable for planting in field conditions.

Acknowledgments

This research was funded by the Ministry of Science and Higher Education of Kazakhstan Republic, Program numbers BR21882024, BR18574099.

We sincerely thank Alina Zemtsova for her invaluable assistance in preparing this paper.

Conflict of interest

All authors are aware of the article's content and declare no conflict of interest.

References

Food and Agriculture Organization of the United Nations., (from February 27, 2025 accessed on March 31.2025). Crops and Livestock Products. Production in 2022. Available online: https://www.fao.org/faostat/en/#data/QCL

2. Jennings D.L. (1988) Raspberries and Blackberries, Their Breeding, Diseases and Growth; San Diego: Acad. Press Ltd, London UK, 230 p. ISBN 978-0123842404

3. Dai J., Gupte A., Gates L., Mumper R.J., A comprehensive study of anthocyanin containing extracts from selected blackberry cultivars: extraction methods, stability, anticancer properties and mechanisms. *Food Chem. Toxicol*. 2009, vol 47, pp 837-847. https://doi.org/10.1016/j.fct.2009.01.016

4. Legrand T., (2018) Overviews of food systems and agroindustry, value chains, and food loss and waste in the countries of eastern Europe and central ASIA. Food and Agriculture Organization of the United Nations Budapest, 75 p. ISBN 978-92-5-130620-8

5. Mudasir I., Singh K.K. Propagation of Temperate Fruit Crops. *In book* 2020, *Innovative Agriculture and Botany Publisher* pp.119-135

6. Reed B., Poothong S.H., Hall K.H. Propagation of blackberries and related *Rubus* species. Blackberries and their hybrids *in book* 2017, pp 101-112 https://doi.org/10.1079/9781780646688.0101

7. Roberto S.R., Colombo R.C. Innovation in Propagation of Fruit, Vegetable and Ornamental Plants. *Horticulturae 2020*, vol 6, p 23-31. https://doi.org/10.3390/horticulturae6020023

8. Romadanova N.V., Kushnarenko S.V. Conservation of plant biodiversity by biotechnology methods Proceedings in Applied Botany, *Genetics and Breeding*. – 2023, vol 184(1), pp 239-248. https://doi.org/10.30901/2227-8834-2023-1-239-248

9. Reed B.M., Poothong S., Hall H.K. Propagation of blackberries and related species. In Blackberries. *CABI: Wallingford, UK*, 2017; pp 101-112. https://doi.org/10.1016/j.fct.2009.01.016

10. Tashmatova L.V., Matsneva O.V., Khromova T. M., Shakhov V.V. (2021) Optimization of individual elements of clonal micropropagation of fruit and berry crops in the production system of healthy planting material. International scientific and practical conference fundamental and applied research in biology and agriculture: current issues, achievements and innovation. Farba, P 8.

11. Dobrenkov E.A., Semenova L.G., Dunaeva S.E., Ukhatova Yu.V. Adaptation of test tube blackberry plants to field environmental conditions. *Proc. Appl. Bot. Genet. Breed.* 2017, vol 178, pp 24-30. https://doi.org/10.30901/2227-8834-2017-1-24-30 (in Russian)

12. Strik B.C., Finn C.E. Blackberry production systems a worldwide perspective. *ActaHortic*. – 2012, vol 946, pp 341-347. https://doi.org/10.17660/ActaHortic.2012.946.56

13. Ružić D., Lazić T. Micropropagation as means of rapid multiplication of newly developed blackberry and black currant cultivars. *Agric. Conspec*. Sci. 2006, vol 71, pp 149-153.

14. Romadanova N.V., Mishustina S.A., Gritsenko D.A., Omasheva M.Y., Galiakparov N.N., Reed B.M., Kushnarenko S.V. Cryotherapy as a method for reducing the virus infection of apples (*Malus sp.*) // *Cryo Letters. Londo*n, 2016. vol. 37(1). – P 1-9. https://www.ncbi.nlm.nih.gov/pubmed/26964019

15. Kushnarenko S.V., Romadanova N.V., Aralbayeva M.M., Zholamanova S.Z., Alexandrova A.M., Karpova O. Combined ribavirin treatment and cryotherapy for efficient *Potato virus M* and *Potato virus S* eradication in potato (*Solanum tuberosum* L.) *in vitro* shoots // *In Vitro Cell. Dev. Biol. – Plant*. 2017. vol 53(4). – P. 425-432. https://doi.org/10.1007/s11627-017-9839-0

16. Martin R.R., Macfarlane S., Sabanadzovic S., Quito D., Poudel B., Tzanetakis I.E. Viruses and virus diseases of *Rubus*. *Plant Dis*. 2013, vol 97, pp 168-182. http://dx.doi.org/10.1094 /PDIS-04-12-0362

17. Strik B., Martin R.R. Impact of *Raspberry bushy dwarf virus* on 'Marion' blackberry. *Plant Dis*. 2003, vol 87, pp 294-296. https://doi.org/10.1094/PDIS.2003.87.3.294

18. Medina C., Matus J.T., Zuniga M., San-Martin C., Arce-Johnson P. Occurrence and distribution of viruses in commercial plantings of *Rubus*, *Ribes* and *Vaccinium* species in Chile. *Cienc. Investig. Agrar*. 2006, vol 33, pp 23-28. https://doi.org.10.7764/rcia.v33i1.324

19. Barbara D.J., Morton. A., Knight. V.H. Occurrence and distribution of *Raspberry bushy dwarf virus* in commercial raspberries plantations in England and Wales. *Acta Hortic.* 2001. vol 551, pp 23-26. https://doi.org/10.17660/ActaHortic.2001.551.2

20. Spak J., Kubelkova D. Epidemiology of *Raspberry bushy dwarf virus* in the Czech Republic. J. *Phytopathol*. 2000, vol 148, pp 371-377. https://doi.org.10.1046/j.1439-0434.2000.00513.x

21. Valasevich N., Kukharchyk N., Kvarnheden A. Molecular characterization of *Raspberry bushy dwarf virus* isolates from Sweden and Belarus. *Arch. Virol*. 2011, vol 156, pp 369-374. https://doi.org/10.1007/s00705-010-0912-9

22. Chamberlain C.J., Kraus J., Kohnen P.D., Finn C.E., Martin R.R. First report of *Raspberry bushy dwarf virus* in *Rubus* multi bracteates from China. *Plant Dis.* 2003, vol 87, pp 603. https://doi.org.10.1094/PDIS.2003.87.5.603A

23. Wood G.A., Hall H.K. Source of *Raspberry bushy dwarf virus* in *Rubus* in New Zealand, and the indefectibility of some newer cultivars to this virus. New Zeal. J. *Crop Hort. Sci*. 2001, vol 29, pp 177-186. https://doi.org.10.1080/01140671.2001.9514176

24. Kolchenko M., Kapytina A., Kerimbek N., Pozharskiy A., Nizamdinova G., Khusnitdinova M., Taskuzhina A., Gritsenko D. Genetic characterization of *Raspberry bushy dwarf virus* isolated from red raspberry in Kazakhstan. *Viruses* 2023, vol 15, pp 975. https://doi.org/10.3390/v15040975

25. Martin R.R., Tzanetakis I.E., Gergerich R., Fernández, G., Pesic, Z. *Blackberry yellow vein associated virus*: a new crinivirus found in blackberry. *Acta Hortic. 2001,* vol 656, pp 137-142. https://doi.org/10.17660/ActaHortic.2004.656.21

26. Halgren A., Tzanetakis I.E., Martin R.R. 2007. Identification, characterization, and detection of *Black raspberry necrosis virus. Phytopathology*. 2007, vol 97, pp 44–50. https://doi.org.10.1094/PHYTO-97-0044

27. Reed B.M., Tanprasert P. Detection and control of bacterial contaminants of plant tissue cultures. A review of recent literature. *Plant Tissue Cult. Biotechnol*. 1995, vol 1, pp 137–142.

28. Orlikowska T., Nowak K., Reed B. Bacteria in the plant tissue culture environment. *Plant Cell Tiss. Org. Cult*. 2017, vol 128, pp 487–508. https://doi.org/10.1007/s11240-016-1144-9

29. Romadanova N.V., Aralbayeva M.M., Zemtsova A.S., Alexandrova A.M., Kazybayeva S. Zh., Mikhailenko N.V., Kushnarenko S.V., Bettoni J.C. *In Vitro* collection for the safe storage of grapevine hybrids and identification of the presence of *Plasmopora viticola* Resistance Genes // *Plants*. 2024, 13, 1089. https://doi.org/10.3390/plants13081089

30. Kaluzna M., Mikicińsk A., Sobiczewski P., Zawadzka M., Zenkteler E., Orlikowska T. Detection, isolation, and preliminary characterization of bacteria contaminating plant tissue cultures. *Acta Agrobot*. 2013, vol 66, pp 81-92. https://doi.org/10.5586/aa.2013.054

31. Tanprasert P., Reed B.M. Determination of minimal bactericidal and effective antibiotic treatment concentrations for bacterial contaminants from micropropagated strawberries. *In Vitro Cell. Dev. Biol. – Plant*. 1997, vol 33, pp 227-230. http://dx.doi.org/10.1007/s11627-997-0027-5

32. Reed B.M., Mentzer J., Tanprasert P., Yu X. Internal bacterial contamination of micropropagated hazelnut: identification and antibiotic treatment. *Plant Cell. Tiss. Org. Cult*. 1998, vol 52, pp 67-70. https://doi.org/10.1023/A:1005989000408

33. George M.W., Tripepi R.R. Plant Preservative Mixture™ can affect shoot regeneration from leaf explants of *chrysanthemum*, European birch, and rhododendron. *HortScience* 2001, vol 36, pp 768-769. https://doi.org/10.21273/HORTSCI.36.4.768

34. Ho W.J., Huang Y.K., Huang W.W., Huang Y.C., Chung J.P. Effective *in vitro* culture using dormant buds of nodal sections from a mature *Acacia* tree. *In Vitro Cell. Dev. Biol*. – *Plant.* 2021, vol 58, pp 437-446. https://doi.org/10.1007/s11627-021-10235-8

35. Romadanova N.V., Tolegen A.B., Koken T.E., Nurmanov M.M., Kushnarenko S.V. Chemotherapy of *in vitro* apple shoots as a method of virus's eradication // *International Journal of Biology and Chemistry* 2021, vol 14, p 5. https://doi.org/10.26577/ijbch.2021.v14.i1.04

36. Kushnarenko S., Aralbayeva M., Rymkhanova N., Reed B.M. Initiation pretreatment with Plant Preservative Mixture™ increases the percentage of aseptic walnut shoots. *In Vitro Cell. Dev. Biol. – Plant.* 2022, vol 58, pp 964-971. https://doi.org/10.1007/s11627-022-10279-4

37. Murashige T., Skoog F., A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant* 1962, vol 15, pp 473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

38. Viss P.R., Brooks E.M., Driver J.A. A simplified method for the control of bacterial contamination in woody plant tissue culture. *In Vitro Cell. Dev. Biol*. 1991, vol 27, pp 42. https://doi.org/10.1007/BF02632060

39. Choudhary S.B., Kumar M., Chowdhury I., Singh R.K., Pandey S.P., Sharma H.K., Karmakar P.G. An efficient and cost effective method of RNA extraction from mucilage, phenol and secondary metabolite rich bark tissue of tossa jute *(C.olitorius L.*) actively developing phloem fiber. *Biotech.* 2016 vol 3, 6(1), p 100. https://doi.org/10.1007/s13205-016-0415-9

40. Constable F.E., Bottcher C., Kelly G., Nancarrow N., Milinkovic M., Persely D.M., Rodoni B.C. (2010) The seasonal detection of strawberry viruses in Victoria, Australia. 21st International Conference on Virus and other Graft Transmissible Diseases of Fruit Crops, Julius Kuhn Archiv. pp 27-34.

41. Gambino G. Perrone I., Gribaudo I. A rapid and effective method for RNA extraction from different tissues of grapevine and other woody plants. *Phytochem. Anal*. 2008, vol 19, pp 520-525. https://doi.org/10.1002/pca.1078

42. Jennings D.L. Raspberries and Blackberries: Their Breeding, Diseases and Growth. San Diego: *Acad. Press Ltd, London UK*, 1988. – pp 230.

43. Kokko H.I., Kivineva M., Kärenlampi S.O. Single-step immunocapture-PCR in the detection of *Raspberry bushy dwarf virus*. *Biotechniques.* 1996, vol 20, pp 842-846. https://www.tandfonline.com/doi/abs/10.2144/96205st03

44. SYSTAT. SYSTAT 13.0, Statistics Software; SYSTAT Software, Inc.: San Jose, CA, USA, 2009; Available online: https://systatsoftware.com/ (accessed on 17 September 2024).

45. Romadanova N.V., Mishustina S.A., Matakova G.N., Kuhsnarenko S.V., Rakhimbaev I.R., Reed B.M. *In vitro* collection of *Malus* shoots cultures for cryogenic bank development in Kazakhstan // *Acta Horticulturae,* March 2016, vol 1113, pp 271-277. http://dx.doi.org/10.17660/ActaHortic.2016.1113.40

46 Lumsden P.J., Nicholas J.R., Davies W.J. Latent bacterial infections: epiphytes and endophytes as contaminants of micropropagated plants; *Springer Nature: Cham, Switzerland*, 1994, pp 379-396. https://doi.org/10.1007/978-94-011-0790-7_43

47. Volk G.M., Bonnart R., Araújo de Oliveira A.C., Henk A.D. Minimizing the deleterious effects of endophytes in plant shoot tip cryopreservation. *Appl. Plant Sci.* 2022, vol 10, e11489. https://doi.org/10.1002/aps3.11489

48. Grimaldi F., Bastos F.E.A. Control of *in vitro* contamination during the establishment of *Pyrus* communis explants using Plant Preservative Mixture™ Plant *Cell Culture & Micropropagation* 2023, vol 19 pp 185-191. https://doi.org/10.46526/pccm.2023.v19.185

49. Kushnarenko S., Aralbayeva M., Rymkhanova, N., Reed B.M. Initiation pretreatment with Plant Preservative MixtureTM increases the percentage of aseptic walnut shoots. *In Vitro Cell. Dev. Biol. – Plant.* 2022, vol 58, pp 964-971. https://doi.org/10.1007/s11627-022-10279-4

50. Orlikowska T., Zawadzka M., Zenkteler E. Sobiczewski P. Influence of the biocides PPM™ and *in vitro* fural on bacteria isolated from contaminated plant tissue cultures and on plant microshoots grown on various media. *J. Hortic. Sci. Biotechnol.* 2015, vol 87, pp 223-230. https://doi.org/10.1080/14620316.2012.11512856

51. Paul A.L., Semer C., Kucharek T., Ferl R.J. The fungicidal and phytotoxic properties of benomyl and PPM in supplemented agar media supporting transgenic arabidopsis plants for a space shuttle flight experiment. *Appl. Microbiol. Biotechnol*. 2001, vol 55, pp 480-485. https://doi.org/10.1007/s002530000521

52. Romadanova N.V., Tolegen A.B., Kushnarenko S.V., Zholdybayeva E.V., Bettoni J.C. Effect of Plant Preservative Mixture™ on endophytic bacteria eradication from *in vitro* grown apple shoots. *Plants* 2022, 11, 2624. https://doi.org/10.3390/plants11192624

53. Thomas P., Agrawal M., Bharathkumar C.B. Use of Plant Preservative Mixture™ for establishing *in vitro* cultures from field plants: Experience with papaya reveals several PPM™ tolerant endophytic bacteria. *Plant Cell Reports* 2017, vol 36 pp 1717-1730. https://doi.org/10.1007/s00299-017-2185-1

Information about authors

Arman Tolegen – PhD-student, M.Sc., Institute of Plant Biology and Biotechnology (Almaty, Kazakhstan, e-mail: tolegenarman7@gmail.com)

Natalya Romadanova – Candidate of Biological Sciences, Associate Professor, Institute of Plant Biology and Biotechnology (Almaty, Kazakhstan, e-mail: nataromadanova@gmail.com)

Moldir Aralbayeva – M.Sc., Institute of Plant Biology and Biotechnology (Almaty, Kazakhstan, e-mail: berim.moldir@mail.ru)

Nazgul Rymkhanova – PhD-student, M.Sc., Institute of Plant Biology and Biotechnology (Almaty, Kazakhstan, e-mail: nazka_0993@mail.ru)

Yulia Ukhatova – Candidate of Biological Sciences, Federal Research Center N.I. Vavilov All-Russian Institute of Plant Genetic Resources(Saint Petersburg, Russia, e-mail: y.ukhatova@vir.nw.ru)

Magamedgusein Agahanov – Candidate of Biological Sciences, Federal Research Center N.I. Vavilov All-Russian Institute of Plant Genetic Resources (Saint Petersburg, Russia, e-mail: m.agahanov@vir.nw.ru)

Natalya Mikhailenko – B.Sc., Master-student, Institute of Plant Biology and Biotechnology (Almaty, Kazakhstan, e-mail: georgi-nata@mail.ru)

Svetlana Kushnarenko – Candidate of Biological Sciences, Professor, Institute of Plant Biology and Biotechnology (Almaty, Kazakhstan, e-mail: sv.kushnarenko.bio@gmail.com)