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Original Article

Micropropagation of berry crops for creation of germplasm cryobanks

Micropropagação de fruteiras para criação de criobancos de germoplasma

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Abstract

One of the main stages of cryopreservation of meristematic tissues in vegetative plants is a clonal micropropagation, which includes isolating the explants of the raw material *in vitro* and optimizing the culture medium for micropropagation. As the result of our studies, the optimal periods for *in vitro* micropropagation are: first – isolation of explants from initiated shoots of dormant buds (blackcurrants and raspberries) in January-March; the second – from actively growing shoots (blackcurrants and raspberries) in May-June, from the formed mustache (strawberry) in July-August. The optimal drugs for sterilization of raspberry explants are: a) 0.1% $HgCl_2$ (6 min), then 3% H_2O_2 (15 min); b) chlorine-containing bleach «Domestos» in the dilution of H_2O 1:9 (10 min). For blackcurrant: a) 0.1% $HgCl_2$ (5 min) in combination with 0.1% fungicide "Topaz" (30 min); b) 0.1% $HgCl_2$ (5 min) in combination with the treatment with $KMnO_4$ (30 min); c) "Domestos" in the dilution of H_2O 1:5 (20 min). For strawberry: a) 0.1% $HgCl_2$ (6 min) followed by treatment with 3% H_2O_2 10 (min); b) 1% deochlor (7 min), 3% H_2O_2 (10 min); c) "Domestos" in the dilution of H_2O 1:5 (8 min) with subsequent treatment 0,1% $HgCl_2$ –7 min, then 0,20 mg/l $KMnO_4$ – 30 min. Optimal compositions of culture media for micropropagation of blackcurrant – Murashige and Skoog (MS) medium with 0.5 mg L^{-1} BAP, 0.5 mg L^{-1} GA_3, 0.1 mg L^{-1} IBA and 20 g L^{-1} glucose. For raspberry –MS medium with 0.5 mg L^{-1} BAP, 0.1 mg L^{-1} IBA, 10 mg L^{-1} iron chelate and 30 g L^{-1} sucrose. For strawberry – MS medium with 0.3 mg L^{-1} BAP, 0.01 mg L^{-1} IBA, 0.2 mg L^{-1} GA_3, 10 mg L^{-1} iron chelate and 30 g L^{-1} sucrose. Based on these studies, the cryobank was created, which include the germplasm of *in vitro* meristematic tissues in 66 cultivars, hybrids and wild-growing forms of blackcurrant, raspberry and strawberry. Therefore, the aim of the research was to obtain aseptic plants, clonal micropropagation and the creation of a cryogenic collection of germplasm based on the developed technology.

Keywords: berry crops, micropropagation, cryobank, germplasm.

Resumo

Uma das principais etapas da criopreservação de tecidos meristemáticos em plantas vegetais é a micropropagação clonal, que inclui o isolamento dos explantes da matéria-prima *in vitro* e a otimização do meio de cultura para micropropagação. Como resultado deste estudo, os períodos ideais para micropropagação *in vitro* são: primeiro – isolamento de explantes de brotos iniciados de gemas dormentes (groselhas e framboesas) em janeiro a março; o segundo – de brotos em crescimento ativo (groselhas e framboesas) em maio a junho, do bigode formado (morango) em julho a agosto. As drogas ideais para esterilização de explantes de framboesa são: a) $HgCl_2$ 0,1% (6 min), depois H_2O_2 3% (15 min); b) lixívia contendo cloro "Domestos" na diluição de H_2O 1:9 (10 min). Para groselha preta: a) $HgCl_2$ a 0,1% (5 min) em combinação com fungicida "Topaz" a 0,1% (30 min); b) $HgCl_2$ 0,1% (5 min) em combinação com o tratamento com $KMnO_4$ (30 min); c) "Domestos" na diluição de H_2O 1:5 (20 min). Para morango: a) 0,1% $HgCl_2$ (6 min) seguido de tratamento com 3% H_2O_2 10 (min); b) dicloro a 1% (7 min), H_2O_2 a 3% (10 min); c) "Domestos" na diluição de H_2O 1:5 (8 min) com tratamento posterior 0,1% $HgCl_2$ – 7 min, depois 0,20 mg/l $KMnO_4$ – 30 min. Composições ótimas de meios de cultura para micropropagação de groselha negra – meio Murashige e Skoog (MS) com 0,5 mg L-1 BAP, 0,5 mg L-1 GA^3, 0,1 mg L^{-1} IBA e 20 g L^{-1} de glicose. Para meio framboesa – MS com 0,5 mg L^{-1} de BAP, 0,1 mg L^{-1} de AIB, 10 mg L^{-1} de quelato de ferro e 30 g L^{-1} de sacarose. Para morango – meio MS com 0,3 mg L^{-1} BAP, 0,01 mg L-1 IBA, 0,2 mg L-1 GA3, 10 mg L^{-1} quelato de ferro e 30 g L-1 sacarose. Com base nesses estudos, um criobanco foi criado incluindo o germoplasma de tecidos meristemáticos *in vitro* em 66 cultivares, híbridos e formas silvestres de groselha, framboesa e morango. Portanto, o objetivo da pesquisa foi a obtenção de plantas assépticas, micropropagação clonal e a criação de uma coleção criogênica de germoplasma com base na tecnologia desenvolvida.

Palavras-chave: culturas de bagas, micropropagação, criobanco, germoplasma.

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1. Introduction

Plant genetic resources are of great scientific and practical value. Cultural plantations and wild forms of berry crops grow on the territory of Kazakhstan, including raspberries (*Rubus idaeus* L.), black currants (*Ribes nigrum* L.) and strawberries (*Fragaria* L.), adapted to local conditions, resistant to pests and diseases (Astley, 2018; Halewood et al., 2018; Panis et al., 2020). However, under the influence of environmental and anthropogenic factors, the areas of their growth are decreasing (Schlaepfer et al., 2018; Swift et al., 2019; González et al., 2020; Mayerhofer et al., 2021). In modern genebanks, to preserve plant genetic resources, along with traditional methods, biotechnological ones are successfully used, which include preserving plant germplasm in culture *in vitro* and cryopreservation at a temperature of -196°C (Dantas et al., 2018; Popova, 2018; Sofi et al., 2020; Özbek and Zencirci, 2021). Cryopreservation of germplasm of plant material in liquid nitrogen guarantees long-term preservation of the material and at the same time reduces the availability of the occurrence of genetic changes possible in *in vitro* culture at positive temperatures (Jenderek and Reed, 2017; Kalaiselvi et al., 2017; Kaya et al., 2017). The technique of cryopreservation of plant tissues includes several stages (Burritt, 2012; Wolkers and Oldenhof, 2015; Linde et al., 2018; Oliveira et al., 2022). Primary explants are selected, isolated, sterilized from saprophytic microflora and aseptic plants are obtained *in vitro*, then optimal nutrient media, cultivation conditions for the growth and development of the explant are selected and multiplied to a sufficient amount for cryofreezing (Hamill and Rames, 2016). After that, the tops of the shoots of aseptic plants are cryo-frozen (Rueda et al., 2015; Maslova and Kuzmina, 2021). The necessary components of nutrient media are sources of mineral nutrition (salts of various macro- and microelements, carbohydrates (sucrose, glucose), biologically active substances (phytohormones), vitamins (Arguelles, 2020). Auxins and cytokinins play a special role in the regulation of growth and development processes (Bielach et al., 2017; Jing and Strader, 2019; Petrasek et al., 2019; Sharma and Zheng, 2019).

One of the main stages of cryopreservation of meristematic tissues in vegetative plants is a clonal micropropagation, which includes isolating the explants of the raw material in vitro and optimizing the culture medium for micropropagation. The aim of the research was to obtain aseptic plants, clonal micropropagation and the creation of a cryogenic collection of germplasm based on the developed technology. In section 2 we have presented the material and methods necessary to carry out this study including details on the plant material used, clonal micropropagation and cryopreservation. Section 3 includes the results obtained throughout the research and finally section 4 gives a summary of the study presented and the acquired results.

2. Materials and Methods

2.1. Plant material

Black currant: wild form - *Ribes nigrum* L., 12 varieties and 2 hybrids. Raspberry: wild forms - *Rubus idaeus* L., *Rubus slesvicensis* Lange, 13 varieties and 10 hybrids. Strawberries: 27 varieties and 1 hybrid.

2.2. Clonal micropropagation

The introduction into the culture *in vitro* was carried out in different periods of the growing season. The overgrown shoots and tops of actively growing shoots of black currant and raspberry with a meristematic zone, and the tips of the strawberry whiskers were sterilized from saprophytic microflora by washing in soapy water and sterile water, followed by treatments with various concentrations of $HgCl_2$, sodium salt of dichloroisocyanuric acid "Deochloride", bleaches containing calcium hypochlorite - "ACE", "Domestos", as well as hydrogen peroxide, fungicides - "Topaz", "Fundazol". Optimization of the nutrient medium composition for clonal micropropagation and restoration of the viability of meristems after cryopreservation was carried out on various modifications of the Murashige and Skoog medium (MS), the composition of which differed in the concentration and composition of carbohydrates (sucrose or glucose), growth stimulants: 6-benzylaminopurine (BAP), β-indolyl-3-butyric acid (IBA), β-indoleacetic acid (IAA) and gibberellic acid (GA) and some mineral components — iron chelate sodium ferric ethylenediaminetetraacetate (NaFe EDTA). The nutrient media were poured into culture tubes (Magenta GA7), 40.0 ml each and sterilized in an autoclave (TYUMEN, VK-75-01) at a pressure of 0.8-1.0 atmospheres for 25 minutes. Testing for the presence of latent bacterial and fungal microflora was carried out on VISS medium (sucrose-10.0 g/l; casein hydrolyzate-8.0 g/l; yeast extract-4.0 g/l; KH_2PO_4; $MgSO_4 \cdot 7H_2O$; gelrite- 6.0 g/l, pH-6.9).

The multiplication factor, average for 1 passage for each genotype, was calculated by the formula: P=a/10b•c; (a - the number of newly formed shoots; b - the number of shoots planted for reproduction; c - the number of passages).

2.3. Cryopreservation

Aseptic plants, grown on the nutrient medium of Murashige and Skoog, were recultivated on fresh nutrient medium every 3 weeks. At the end of a 3-week cultivation cycle, the plants were placed in a climatic chamber (Lab-Line Environette, Melrose Park, IL, USA) for adaptation to cold for 3 weeks at an alternating mode: 8 h +22 °C (illumination intensity 10 µmol $m^{-2}s^{-1}$), then 16 hours –1°C (without illumination).

For cryopreservation of raspberry meristematic tissues, the method of vitrification with pre-cultivation on a medium with 0.3M sucrose developed by Matsumoto et al. (1994), modified by Kovalchuk et al. (2010) was used.

For black currant - methods of vitrification with pre-cultivation on a medium with 0.3 M sucrose and encapsulation-dehydration (Dereuddre et al., 1990), modified by Reed (2002), and Kovalchuk et al. (2017). For strawberries - the method of encapsulation-dehydration.

The apical buds of aseptic plants of black currant and raspberry of 800-1000 microns in size, of strawberries - 600-800 microns with 3-4 leaf primordia were cryopreserved. The experiments were carried out in

3 replicates (n = 60), 65 meristems of each sample were isolated, of which 5 served as control (without freezing in liquid nitrogen), 60 meristems in three cryotubes (20 pcs each) passed all stages of cryopreservation, one cryotest with meristems were thawed after 15-20 minutes to determine the regeneration capacity, the other two from each sample were left for long-term storage in a cryobank. The assessment of the viability of the meristems after cryopreservation was determined within six weeks by the dynamics of growth and development on special nutrient media for recovery.

Statistical analysis was performed in accordance with the standard ANOVA procedures in the SYSTAT software package in 2007.

3. Results and Discussion

Sterilization from saprophytic bacterial and fungal infection of isolated tissues from growing *ex situ and in situ* plants into *in vitro* culture is an extremely important stage of clonal micropropagation. As a result of the research, the optimal treatments for sterilizing raspberry explants were determined: a) 0.1% $HgCl_2$ - 6 min followed by treatment with 3% H_2O_2 - 15 min (regeneration of shoots 95.0%); b) chlorine-containing bleach "Domestos" in a dilution of H_2O 1:9 - 10 min (60.0%). Sterilization is effective for black currant: a) 0.1% $HgCl_2$ - 5 min, then 0.1% "Topaz" fungicide - 30 min (85.7%); b) 0.1% $HgCl_2$ - 5 min, then 0.20 mg/l $KMnO_4$ - 30 min (87.5%); c) "Domestos" in a dilution of H_2O 1:5 - 20 min (85.7%). For strawberries: a) 0.1% $HgCl_2$ - 6 min, then 3% H_2O_2 - 10 min (87.0%); b) 1% "Deochlorom" - 7 minutes, then 3% H_2O_2 - 10 minutes (80.0%); c) "Domestos" diluted with H_2O 1:5 - 8 min, followed by treatment with 0.1% $HgCl_2$ –7 min, then 0.20 mg/l $KMnO_4$ - 30 min (87.5%). The duration of treatment depended on the varieties introduced into the aseptic culture. The use of other sterilization regimens and preparations led either to necrosis of the explants, or to the manifestation of bacterial and fungal infections and the subsequent death of the apexes. Testing for latent infection on the VISS provocative medium showed 5.0 to 20% of isolated black currant shoots, 5.0 to 13.0% of raspberries and 8.0 to 20% of strawberries remain infected and must be discarded.

The composition of the culture medium is of great importance for the initiation of growth and propagation *in vitro*. In numerous publications devoted to research on cloning of fruit and berry crops, the culture medium of Murashige and Skoog is used. However, a number of authors indicate the need to optimize the content of growth regulators and their concentrations in the composition of nutrient media for specific varieties and laboratory conditions (Kovalchuk et al., 2017). The influence of various phytohormones and their concentrations in the medium of MS on the growth, development and ability to reproduce in artificial conditions of strawberries, raspberries and black currants was studied. Figure 1 shows some of the most representative experimental results.

The ability of varieties to clonal micropropagation is different, raspberry and strawberry plants under artificial conditions reproduce more intensively than black currant. For clonal micropropagation of raspberries, MS medium containing 10 mg/l NaFe EDTA, 0.5 mg/l BAP, 0.1 mg/l IBA, 30 g/l sucrose is optimal. For strawberries - MS medium containing 10 mg/l NaFe EDTA, 0.3 mg/l BAP, 0.01 mg/l IBA, 0.2 mg/l GA, 30 g/l sucrose. For black currant - MS medium containing 5 mg/l NaFe EDTA, 0.5 mg/l BAP, 0.5 mg/l GA; 0.1 mg/l IBA, 20 g/l glucose. While the optimal content of the medium for micropropagation of raspberries is 0.1 mg/l BAP and 0.1 mg/l IBA (Reed, 2002). For black currant, the recommended content of 0.75-1.0 mg/l BAP and 0.5 mg/l IAA (Belevtsova et al., 2021), and for strawberries, - 0.5-2.0 mg/l BAP and 0.5-3.0 mg/l IBA (Plaksina et al., 2018).

Three weeks after passage on a fresh nutrient medium for micropropagation, the plants reached the required

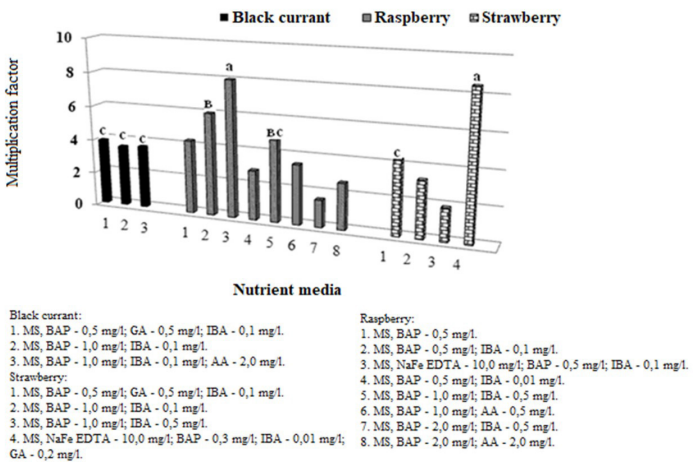

Figure 1. Influence of nutrient media composition on clonal micropropagation of black currant, raspberry and strawberry (average). a, b, c: a significant difference between the results obtained in different treatments of the experiments; bc: treatments of experiments, the results of which do not have significant differences.

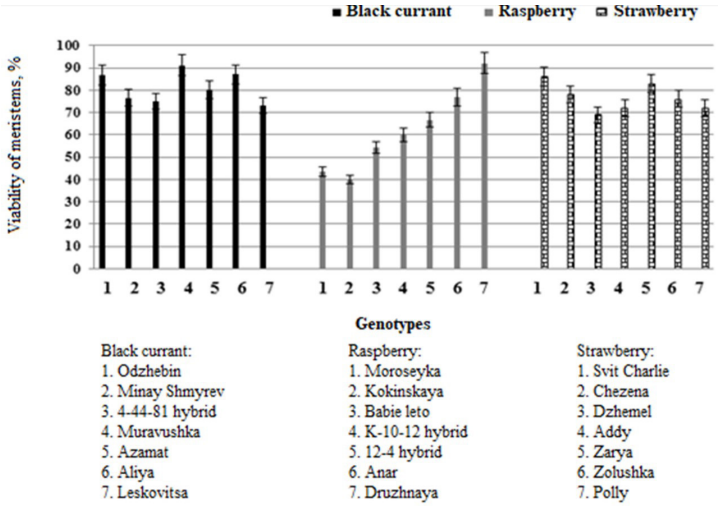

Figure 2. Regenerative capacity of meristematic tissues with long-term storage of germplasm in the cryobank (-196 °C).

developmental stage for isolating apical meristems, cryofreezing and creating a cryobank (shoot height 3-5 cm, stems with pronounced internodes, large leaves with a bright green color, apical shoots with good differentiated meristematic zone).

In the world practice, research is being carried out on the cryopreservation of germplasm and cryobanks of plants are being created. For example, in the United States, cryopreservation, along with traditional methods, is used to preserve genetic material in the cryobank of the National Clonal Germplasm Repository USDA, Oregon, Corvallis; 500 000 samples of commercially valuable, as well as rare and endangered plants belonging to 10 000 species (Volk et al., 2018). The cryobank of germplasm also functions at the Agricultural Research Institute (ARI) of Plant Breeding named after N.I. Vavilov (Dunaeva et al., 2019).

Based on previous studies (Kovalchuk et al., 2010), a cryogenic collection was created, consisting of 66 varieties, hybrids and wild forms of berry crops. Figure 2 shows the results of determining the regenerative capacity of meristematic tissues during long-term storage in a cryobank (–196 °C). The viability of meristems thawed after a year of storage in liquid nitrogen is quite high and exceeds the 40% minimum regeneration limit required for safe storage.

The cryobank contains 60 meristems of each genotype, frozen in three cryotubes, which allows the material to be partially thawed as needed. Cryogenic collection of black currants: wild form - *Ribes nigrum* L., 12 varieties - Azamat, Aliya, Belorusskaya Sladkaya, Willoughby, Katyusha, Leskovitsa, Minay Shmyrev, Muravushka, Pamyat Vavilova, Primorsky Velikan, Odzhebin, Kerry, and 2 hybrids - 4-44-138, 4-44-81. Raspberries: wild forms - *Rubus idaeus* L., *Rubus slesvicensis* Lange, 13 varieties Aray, Anar, Babie Leto, Boskopsky Velikan, Dalnyaya, Druzhnaya, Kokinskaya, Lyulin, Moroseyka, Solokha, Taganka, Shtambovaya, Arlovsky Vals, 10 hybrids - K-10-12, 4/9, 4/33, 4/17, 4/42, M-2-1-18, 12-4, 11-5, 392.001, 459.001. Strawberries of 27 varieties: Dzhemel, Dinamovka, Doch purpurovoy, Zarya, Addi, Zolushka, Cardinal, Konservnaya Rannyaya, Nadezhda, Polly, Troubadour, Ukrainian, Chezena, Frakunda, Yuni, Kokhana, Redgontlit, Svit Charlie, Zenga-Tigayga, Marilva Machat, Senda Cirande, Sumas, Super Festion, Douglas, Hecker, Jucunda, Sparkle, Lord and 1 hybrid 4-23 (BGCI, 2022).

According to the literature, the choice of cryopreservation method depends on the plant genotype. The survival rate of raspberry meristems ranged from 40 to 90%, which is consistent with the literature data (Ukhatova et al., 2017). The proliferation of shoots in aseptic culture is greatly influenced by the components of nutrient media, especially the content and type of carbohydrates and growth regulators, as well as the content of sodium ferric ethylenediaminetetraacetate (NaFe EDTA). In our experiments, we selected the optimal sterilizing agents for *in vitro* administration, their concentration and treatment time. At the same time, it was taken into account that an increase in the exposure time, as well as in the concentration of sterilizing substances, reduces the viability of explants (Ivanova-Khanina, 2014). When selecting media for clonal micropropagation of raspberries, a twofold increase in the content of NaFe EDTA had a good effect, which corresponds to the literature data (Muratova et al., 2020). Replacement of sucrose with glucose in the medium for currants is necessary to eliminate vitrification of shoots.

4. Conclusion

The development of effective methods for plant clonal micropropagation is a basic and necessary condition for creation of genebanks in vitro. Studies on clonal micropropagation and cryopreservation of raspberries, strawberries and black currants have been carried out. It has been established that the most effective method of sterilizing explants when introduced into an aseptic culture is: for raspberries - 0.1% $HgCl_2$ - 6 min, followed by treatment with 3% H_2O_2 - 15 min; for strawberries - "Domestos" diluted with H_2O 1:5 - 8 min, followed

by treatment with 0.1% $HgCl_2$ - 7 min, then 0.20 mg/l of $KMnO_4$ - 30 min, and for black currant - 0.1% $HgCl_2$ - 5 min, then 0.20 mg/l of $KMnO_4$ - 30 min. The optimal composition and concentration of phytohormones, as well as carbohydrates and some minerals in nutrient media for clonal micropropagation have been selected. After obtaining a sufficient number of aseptic plants, cryopreservation of apical meristems was carried out by vitrification with 0.3M sucrose and encapsulation-dehydration. On the basis of the research carried out, a cryogenic collection of germplasm of berry crops has been created, including 13 varieties and 10 hybrids of raspberries, 27 varieties and 1 hybrid of strawberries, 12 varieties and 2 hybrids of black currant, as well as wild forms of black currant and raspberry. In the future, the conditions for cryopreservation of germplasm of other fruit and berry plants will be studied.

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