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Cryopreservation of Ribes nigrum and Ribes rubrum cuttings for long-term storage

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Abstract

The development of fruit growing in Kazakhstan, especially for berry crops, requires planting material for storage and propagation using vegetative methods. There are no industrial areas for blackcurrants and redcurrants in the republic, although the demand for berries and products based on them remains high. The purpose of the study is to investigate the features of cryopreservation of Ribes nigrum and Ribes rubrum cuttings to create a genetic bank for species and varieties and increase the possibilities for their long-term storage for subsequent propagation. Experiments to optimize cryopreservation conditions were carried out at the laboratory “Research Park of Biotechnology and Eco-Monitoring” of Karaganda Buketov University in 2023-2024. The cuttings were frozen in liquid nitrogen in Dewar vessels at a temperature of -196°C. Cuttings of Ribes nigrum and Ribes rubrum of different thicknesses harvested in autumn were used. Freezing was carried out without the use of cryoprotectants and with the use of cryoprotectants (glucose, sucrose, and glucose at concentrations of 20 and 40%). The influence of container type, freezing conditions, thickness of plant materials, and cryoprotectants on the viability of blackcurrant and redcurrant cuttings after storage in liquid nitrogen was investigated. It was found that plastic cryotubes are the best containers for both types of currants. It is better to choose thicker cuttings with a diameter of 6-8 mm, which showed the best survival results after freezing in liquid nitrogen. The highest survival rates for black currant cuttings were obtained with two-stage freezing; no reliable data were obtained for red currants. The use of cryoprotectants significantly increased the survival rate of currant cuttings. The highest survival rate was obtained when using glycerin at a concentration of 40%. Thus, the results of the study show that the optimal conditions for cryopreservation of black and red currant cuttings are plastic containers, thick cuttings 6-8 mm in diameter, the use of two-stage freezing, and a cryoprotectant glycerol at a concentration of 40%. The results obtained were used for long-term storage of currant cuttings and the creation of a genetic bank at Karaganda Buketov University.

Keywords: Cryopreservation, Cryoprotectants, Survivability, Cuttings thickness, Cuttings, Ribes nigrum L., Ribes rubrum L.,Types of containers.

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1. Introduction

The development of agriculture is an important task in Kazakhstan [1]. Currently, about half of all fruit and vegetable products consumed by the population of the republic are imported from abroad (Kyrgyzstan, Uzbekistan, Poland, China, and Belarus). This aspect has a negative impact on product quality, cost, and availability to the population. Part of the demand for fruit and berry crops is met by small-scale private production [2]. Therefore, the question of developing domestic production arises.

Among the valuable and scarce fruit and berry products, blackcurrants and redcurrants are worth mentioning. Currants are a valuable dietary and vitamin source, can be eaten fresh as a dessert, or preserved in the form of jams, compotes, mousses, juices, and nectars [3, 4]. The berries are used as natural colorants in the food industry, as well as ingredients in dietary and therapeutic foods [5].

Ribes nigrum L. (Grossulariaceae family) fruits contain biologically active substances such as sugars (fructose, glucose, sucrose, etc.), organic acids (malic, citric, quinic, and ascorbic), vitamins (B1, B2, B5, B6, C, E), phenolic compounds, anthocyanins, and pectins [6-14]. The content of 19 inorganic elements has been established in the fruits and leaves of black currants, such as Mg, Fe, Mn, K, Na, Ca, Si, Al, Se, Zn, Co, Mo, and others [15].

The complex of phenolic compounds exhibits antioxidant, anti-inflammatory, phytoestrogenic, and hypocholesterolemic activity and helps lower blood sugar levels [16-19]. Vitamins have an antioxidant effect [20] help strengthen the immune system, have a beneficial effect on the nervous system, improve memory, and aid in the biosynthesis of collagen and acids in the production of certain peptide hormones [21]. Polysaccharides in black currants help reduce skin dehydration under the influence of solar radiation and improve immunity [22].

Ribes rubrum L. fruits contain vitamins (A, C, E, B group), antioxidants, anthocyanins, flavonoids, organic acids (malic, fumaric, citric), fiber, and minerals [23-25]. The fruits and juice can be used as a food source, in confectionery, and in the liquor industry. Red currant berries act as an antispasmodic agent [26] strengthen the immune system, improve digestion (peristalsis) and the condition of the cardiovascular system, and have diuretic, choleretic, and anti-inflammatory properties [25, 27]. In folk medicine, the berries are recommended for arthritis, gout, hypovitaminosis, to lower blood sugar levels, and improve vision [28].

However, there is no industrial production of black and red currants in Kazakhstan, and the development of horticulture is hampered by the lack of planting material.

The establishment and expansion of currant plantations must be carried out vegetative way due to the need to preserve the quality of the varietal material [29, 30]. Cuttings are harvested in Central Kazakhstan in July-August, while the optimal planting time is April-May. Traditional methods of storage in refrigerators at low positive temperatures do not fully ensure preservation, as the cuttings dry out and are damaged by fungal and bacterial infections, leading to a loss of viability of up to 50-90% of the harvested cuttings.

Another important issue is ensuring the long-term preservation of plant material, especially for varietal material, when creating germplasm banks [31-34].

There are some downsides to storing fruit and berry plant cuttings as living collections or seed collections [35]. These include: the high cost of maintaining a collection of living plants due to care, watering, protection from diseases and pests, regular transplanting, and measures to preserve varietal characteristics; high level of damage; the need for a large area for a collection of living plants; cross-pollination of plants and loss of varietal qualities; degeneration of varieties during cultivation and reseeding; difficult reproducibility of the method and dependence on soil and climatic conditions; inability to use seeds to maintain the varietal identity of fruit and berry crops.

Modern methods of genetic material preservation include biotechnological methods, such as in vitro collections [36] and cryopreservation of plant germplasm [37, 38].

There are well-established protocols for cryopreservation of seeds, buds, and meristems [39, 40] but methods for freezing cuttings at liquid nitrogen temperatures have not been sufficiently studied. Based on the above, the search for methods for preserving fruit plant cuttings is an important area of research.

Therefore, the present study aims to investigate the characteristics of cryopreservation of *Ribes nigrum* and *Ribes rubrum* cuttings.

2. Materials and Methods

2.1. Collecting Shoots of Red and Black Currants

Lignified shoots of *Ribes nigrum* and *Ribes rubrum* were collected in the third decade of August 2023 from the collection of the Faculty of Biology and Geography of Karagandy Buketov University. Fresh cuttings were dried to a moisture content of 8-10%, stored in a refrigerator at a temperature of +5°C and humidity not more than 50%. The thickness of cuttings with 3-4 buds ranged from 6-8 mm (thick) to 4-5 mm (thin) (Figure 1).

A B

Figure 1.
Shoots of *Ribes nigrum* (A) and *Ribes rubrum* (B).

2.2. Methodology for Cryopreservation of Currant Cuttings

Experiments on the cryopreservation of currant cuttings were conducted in the laboratory "Research Park of Biotechnology and Eco-Monitoring" of Karaganda Buketov University in 2023-2024.

To optimize cryopreservation conditions [39, 41, 42] the following series of experiments were conducted:

i) Freezing of cuttings in different containers (5 ml Falcon plastic cryotubes; Zip-lock foil bags; polyethylene bags);

ii) Shock freezing in liquid nitrogen and two-stage freezing (with preliminary cooling in a freezer at -15° C);

iii) Freezing in liquid nitrogen with cryoprotectants.

The cuttings, packed in containers, were placed in CDC 20 CryoMach Dewar vessels (volume 30 L). Aqueous solutions of sucrose, glucose, and glycerin at concentrations of 20 and 40% were used as cryoprotectants [43-45]. The cuttings were soaked for 10 minutes in cryoprotectant solutions cooled to 0°C, then placed in containers and frozen. All experiments were carried out in 10 repetitions. Cuttings stored in a refrigerator at 0–+2° C served as controls.

The cuttings were thawed after 1 month of storage in liquid nitrogen at a temperature of +20-22° C. The cuttings were washed 3 times with distilled water to remove the cryoprotectants. To activate the cuttings after storage, they were soaked in a 0.5% aqueous solution of potassium humate (Figure 2). Germination was carried out for 10-14 days until the leaf buds opened (Figure 3), which indicated that the cuttings remained viable.

Figure 2.
Thawing of cuttings and soaking in sodium humate solutions.

Figure 3.
Viable and rooted black currant cuttings after cryopreservation

2.3. Method of Analysis

Statistical processing and visualization of experimental data were performed in Statistics 8.1 software. Statistical differences were determined by one-factor 0.05, Tukey'≤ ANOVA analysis of variance.

3. Results

3.1. Viability of Currant Cuttings Depending on the Type of Container

In the first series of experiments, the cuttings were subjected to shock freezing by immersion in liquid nitrogen. Cuttings were not separated by thickness. The results of freezing cuttings of both currant species without the use of cryoprotectants showed different survival rates depending on the type of container (Figure 4).

In the control, the survival rate of cuttings after storage in a refrigerator was 60% for black currants and 80% for red currants. In plastic cryotubes, the survival rate of cuttings was 80% for black currants and 70% for red currants. In foil containers and polypropylene bags, viability decreased by 50% and 30%, respectively.

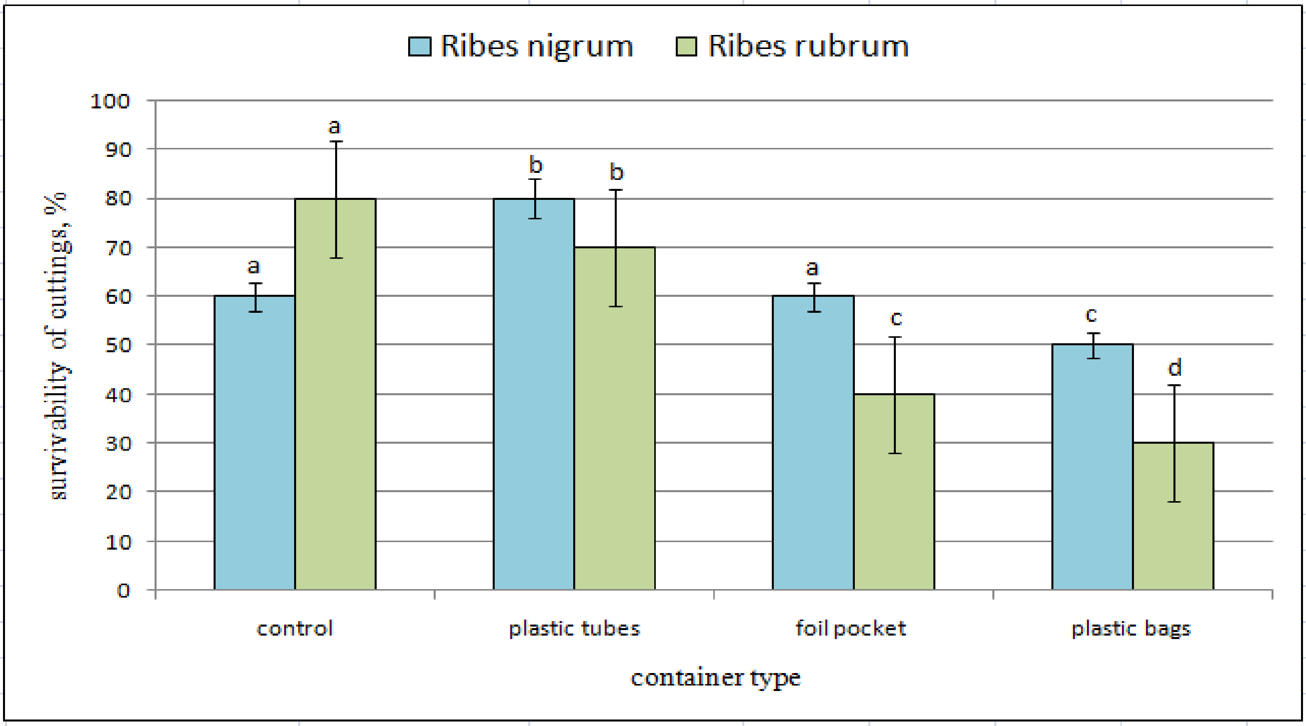

Figure 4.
Survival rate of Ribes nigrum and Ribes rubrum cuttings depending on the type of container used for cryopreservation; a, b, c, d - different letters indicate statistically significant differences between groups (P 0.05).

3.2. Viability of Currant Cuttings Depending on Their Thickness

In the second series of experiments, we evaluated the viability of currant cuttings depending on their thickness. Freezing was carried out in plastic test tubes, with cuttings that were not separated into thick and thin ones serving as a control. The results showed that for both types of currants, thick cuttings (6–8 mm) tolerated freezing in liquid nitrogen better than thin ones (4–5 mm) and exceeded the control results (Figure 5).

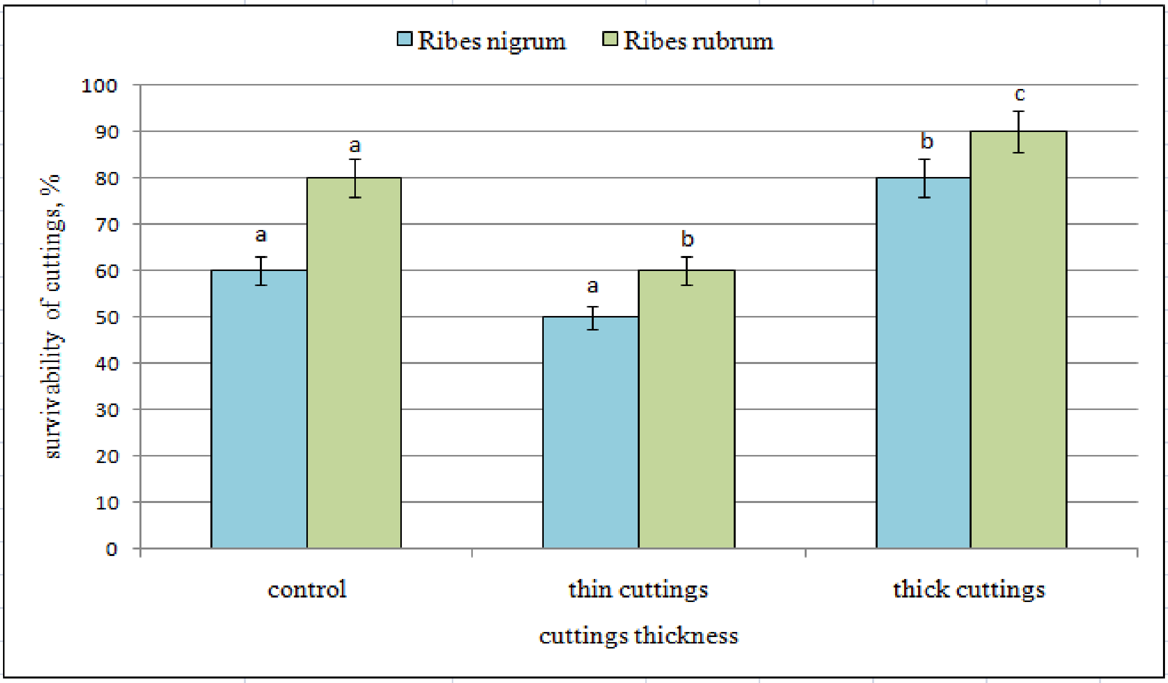

Figure 5.
Survival rate of Ribes nigrum and Ribes rubrum cuttings depending on thickness; a, b, c - different letters indicate statistically significant differences between groups (P 0.05).

3.3. Viability of Currant Cuttings Depending on the Type of Freezing in Liquid Nitrogen

Two types of freezing were used in the experiment: two-stage and shock freezing. The control was a variant of the experiment with storage in a refrigerator (without cryogenic freezing). The results showed that two-stage freezing was more effective than shock freezing (Figure 6).

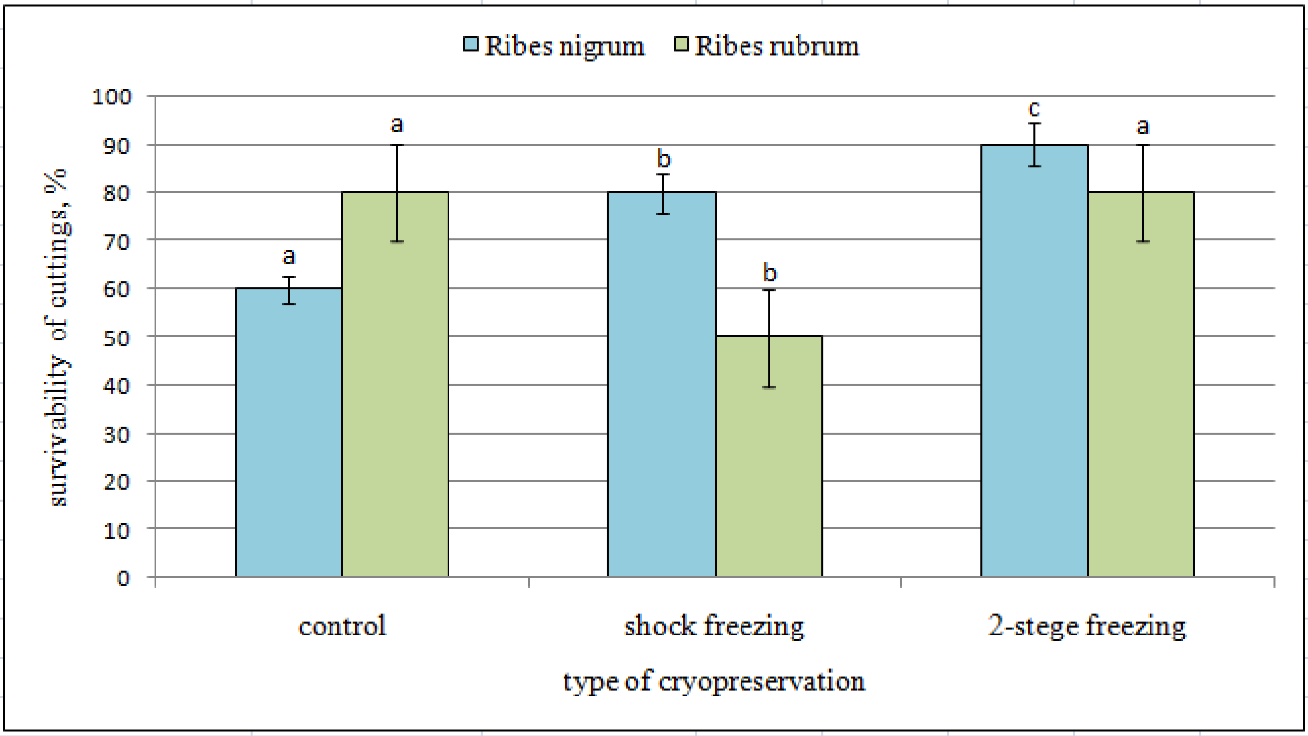

Figure 6.
Survival rate of *Ribes nigrum* and *Ribes rubrum* cuttings depending on type of freezing; a, b, c - different letters indicate statistically significant differences between groups (P 0.05).

3.4. Viability of Currant Cuttings Depending on the Type and Concentration of Cryoprotectant

The results of the study showed that freezing currant cuttings without cryoprotectants resulted in viability after storage in liquid nitrogen ranging from 30 to 90%. Literature data [46, 47] show that the use of cryoprotectants contributes to increased viability.

Figure 7 shows the survival rates of currant cuttings after freezing in liquid nitrogen with sucrose, glucose, and glycerol at concentrations of 20 and 40%. The control was provided by experimental variants with cryopreservation without cryoprotectants.

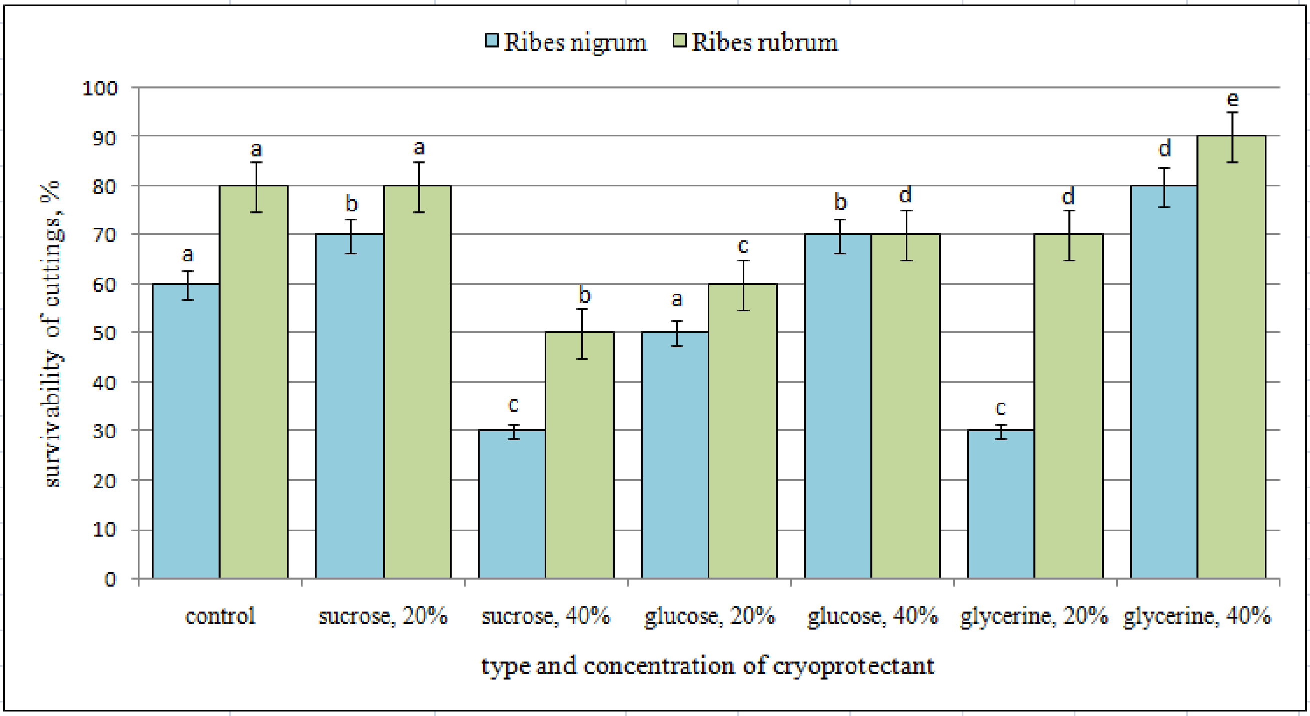

Figure 7.
Survival rate of *Ribes nigrum* and *Ribes rubrum* cuttings depending on type and concentration of cryoprotectant; a, b, c, d, e - different letters indicate statistically significant differences between groups (P 0.05).

4. Discussion

The results obtained showed that black and red currant cuttings can have different survival rates depending on cryopreservation conditions, such as the type of container, freezing conditions, cutting thickness, and the use of cryoprotectants.

The use of different types of containers showed that the best results for black currants were obtained with plastic cryotubes (80%), while the lowest values were obtained with plastic bags (50%). These indicators differed significantly from the control values. The use of foil containers did not show any significant differences from the control variant. For red currants, significantly better survival results were obtained against the control, as survival was lower in all variants of container use during cryopreservation.

A comparison of experiments with thick and thin cuttings showed that in all variants, the survival rate of thick red currant and black currant cuttings was higher than when using cuttings without size separation. For black currants, the maximum survival rates were 80%, and for red currants, 90%, which was significantly higher than the control. The data obtained can be explained by the fact that thicker cuttings have more developed secondary covering tissues, which increases their survival during storage in liquid nitrogen.

Testing of two freezing methods revealed that for black currants, the best results were obtained with two-stage freezing with preliminary cooling in a freezer – 90%, which is significantly higher than the control values (60%) and the survival rate of cuttings during shock freezing (80%). For red currants, shock freezing proved ineffective (50%), which was significantly lower than the control (80%) (freezing without cryoprotectants). Two-stage freezing (80%) did not differ significantly from the control.

The use of cryoprotectants for both types of currants showed that the highest results were obtained with glycerin at a concentration of 40%. For black currants, the survival rate of cuttings was 80%, and for red currants ( ), it was 90%. The other cryoprotectants showed lower results or did not differ significantly from the control.

Thus, the study showed that optimizing cryopreservation conditions for blackcurrant and redcurrant cuttings can increase their survival rate during storage in liquid nitrogen.

5. Conclusion

The results of cryopreservation of *Ribes nigrum* and *Ribes rubrum* cuttings showed a high degree of variability in survival rates depending on the freezing conditions in liquid nitrogen. This allows for the optimization of conditions for long-term storage of cuttings in liquid nitrogen. Plastic cryotubes have been determined to be the optimal container for cryopreservation of blackcurrant and redcurrant cuttings. When cryopreserving cuttings of both currant species, it is preferable to select thick cuttings of 6-8 mm. When choosing a cryopreservation method for black currants, the best results were obtained with a two-stage freezing process, while no significantly higher indicators were found for red currants. The use of cryoprotectants made it possible to identify the highest reliable indicators with glycerol at a concentration of 40% for both black and red currants.

Thus, optimization of cryopreservation conditions for currant cuttings allows the development of a liquid nitrogen freezing protocol to ensure long-term storage.

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