INTRODUCTION OF IN VITRO GRAPES OF INTERSPECIFIC ORIGIN

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Abstract. During clonal micro-propagation of grapes of interspecific origin of the Aleshenkin variety, it was revealed that the optimal type of explants for initiating a sterile culture are meristematic apexes 100 – 150 μm in height, which are advisable to be planted in test tubes on a nutrient medium according to the prescription of Quorin Lepuavr (QL) (Quoirin & Lepoivre, 1977) enriched with the following substances (mg/l): thiamine (B1), pyridoxine (B6), nicotinic acid (PP) – 0.5 each; 6-BAP – 0.1; inositol – 100; sucrose – 30,000, agar-agar – 7,000 and incubate for 70 days in a light room at an illumination intensity of 2,500 lux, a 16-hour photoperiod and a temperature of 20 – 22 C. At the multiplication stage, with two consecutive passages of 40 days of sub-culturing on a nutrient medium according to the prescription of Murashige and Skoog (MS) (Murashige & Skoog, 1962), enriched with the following substances (mg/l): thiamin (B1), pyridoxine (B6), nicotinic acid (PP) – 0.5 each; 6-BAP – 0.1, inositol – 100; sucrose – 30,000, agar-agar – 7,000, the advantage of this type of explants has been shown. Since in terms of the coefficient of multiplication, regenerant plants introduced into culture in vitro by meristematic apexes after the first passage were 2.3 times and after the second passage 1.4 times higher than the plants introduced into culture by micro-cuttings.

1. Introduction
In central Russia, grapes are cultivated relatively recently, since for quite a long time this culture was considered unpromising for the weather conditions of the Nonchernozem Belt (Non-Black Earth Region). The spread of the culture was facilitated by the emergence of new table varieties with a short growing season, the fruits of which have time to ripen in a relatively short summer and give a high yield with good quality berries. [2, 15].

In 2020, in the State Register of Breeding Achievements Admitted to Use for Cultivation in the Central Region of the Russian Federation, 54 grape varieties are recommended, varied in yield, shape, size and taste of fruits [7]. The assortment of modern grape varieties for the Nonchernozem Belt is
mainly interspecific hybrids, often based on *V. amurensis*, *V. riparia*, *V. labrusca*, which entails problems associated with their vegetative reproduction [2, 5, 8].

Therefore, at present, there is a lack of high-quality planting material for grapes of interspecific origin, therefore, one of the promising areas of research in the field of vegetative propagation of this culture is the optimization of the stages of clonal micro-propagation technology [16].

Clonal micro-propagation is a modern intensive method of mass asexual reproduction of plants in tissue and cell culture, in which the resulting plants are genetically identical to the original specimen [2, 9, 13]. When using it, the tissues of micro-shoots are released from the causative agents of many diseases, which reduce the yield to 30 – 80% [18], and rejuvenilization of the organism after culture in *vitro* enhances the ability to vegetative reproduction [21]. The technology of clonal micro-propagation allows for a short period of time to obtain a large amount of planting material, more than a thousand plants per year from one meristem introduced into the culture, which is hundreds of times more than when using traditional methods of vegetative propagation [3].

When the meristematic apexes are introduced into the culture in *vitro*, a rather long period of time passes from the beginning of growth to the development of the meristematic apex into a full-fledged conglomerate of micro-shoots, suitable for micro-cutting. Sometimes, during micro-propagation of plants, already organized structures (axillary buds or micro-cuttings) can be used as explants. It is known that when using large explants, there is a high growth rate of tissues of micro-plants and awakening of buds, active proliferation in subsequent passages and ease of work. [14, 17], however, restrained growth of regenerant plants or latent fungal and bacterial infections may occur. It should be noted that the coefficient of multiplication in micro-plants obtained from large explants is usually lower than in micro-plants obtained from meristematic apexes.

According to the methods generally accepted in the technology of clonal micro-propagation of grapes, the initial explants are planted on a nutrient medium according to the prescription of Murashige and Skoog without the addition of synthetic cytokinins [1, 2]. However, a lot of researchers who have worked with culture in *vitro* point to significant species and even varietal differences in plants in terms of the needs for the mineral and hormonal composition of the nutrient medium and require individual selection of components for effective growth and development of micro-plants [6, 9]. Therefore, it was decided to identify the feasibility of using a nutrient medium for the introduction of experimental explants into a culture in *vitro* according to the prescription of Quorin Lepuavr with the addition of 6-BAP at a concentration of 0.1 mg/l.

The purpose of the research is to identify the optimal type of explants and a nutrient medium that ensures the growth of micro-plants introduced into a culture in *vitro* at the stage of multiplication.

2. Conditions, Materials and Methods

The experiments were carried out in 2019 – 2020 in the departments of biotechnology and grape culture of the fruit growing laboratory of the Russian State Agrarian University – Moscow Agricultural Academy named after K.A. Timiryazev.

The object of research was the Aleshenkin grape variety of interspecific origin. When introduced into a culture in *vitro*, the tops of shoots 2 – 3 cm long were first cut, then they were cleaned with a brush and detergent, then they were washed for 30 minutes under running water, followed by continuous stirring for 15 minutes in a solution of the fungicide Fundazole at a concentration 1 g/l. The explants were sterilized in aseptic conditions in a laminar box, first with alcohol (70%) for 1 – 2 seconds, then with sodium hypochlorite solution (active chlorine content 3%) with 5% anionic surfactants in a dilution of 11 ml per 100 ml of solution for 10 – 15 minutes. Removal of the sterilizing agent was carried out three times by washing the explants in sterile water. Then, under a binocular magnifying glass, the initial explants were isolated and placed in test tubes on a nutrient medium. Lateral buds, meristematic apexes 100 – 150 μm in height with leaf primordia and micro-cuttings 0.5 – 1.0 cm in size were taken as explants.

For the introduction of explants into a culture in *vitro*, a nutrient medium was used according to the prescription of Quorin Lepoiivre (QL) (Quorin & Lepoiivre, 1977) [22] enriched with the following
substances (mg/l): thiamine (B1), pyridoxine (B6), nicotinic acid (PP) – 0.5 each; 6-BAP – 0.1; inositol – 100; sucrose – 30,000, agar-agar – 7,000. As a control, we used a nutrient medium without synthetic hormones (w/h) according to the prescription of Murashige and Skoog (MS) (Murashige & Skoog, 1962) [20] enriched with the following substances (mg/l): thiamine (B1), pyridoxine (B6), nicotinic acid (PP) – 0.5 each; inositol – 100; sucrose – 30,000, agar-agar – 7,000. Further, the cultures were incubated for 70 days in a light room at an illumination intensity of 2,500 lux, a 16-hour photoperiod and a temperature of 20 – 22°C. Repetition of experiments at this stage of research is threefold, 10 tubes in one repetition.

Then, 70 days after the introduction into the culture in vitro, at the stage of multiplication, two consecutive passages were made on the nutrient medium according to the prescription of Murashige and Skoog (MS) [20] enriched with the following substances (mg/l): thiamine (B1), pyridoxine (B6), nicotinic acid (PP) – 0.5 each; 6-BAP – 0.1, inositol – 100; sucrose – 30,000, agar-agar – 7,000. The duration of the subcultivation period was 40 days, records of the morphometric indicators of development and the coefficient of multiplication of regenerant plants were maintained on the 20th and 40th day. In a laminar flow unit, 10 microscopes 2 – 3 nodes long were placed in each vessel. Then the cultures were incubated in a light room at an illumination intensity of 2,500 lux, a 16-hour photoperiod and a temperature of 20 – 22°C.

Repetition of experiments is threefold, 5 plants in one repetition. Statistical processing of the results was carried out according to A.V. Isachkin using Micro-sof Office Excel 2007 [12]. Their application has confirmed the reliability of the results obtained.

3. Results and Discussion

Introduction to culture is the most difficult and costly stage in the technology of clonal micro-propagation, its productivity, as a rule, is not high, and labour costs are very significant. [2, 3].

In addition, since mother plants are ubiquitously infected with a large set of micro-organisms, effective sterilization of plant explants and adherence to aseptic rules do not exclude subsequent bacterial and fungal contamination. The loss of explants from latent bacterial infection is especially noticeable. As the number of passages increases, the proportion of micro-plants with latent bacterial infection increases, which can inhibit regeneration and cause the death of plant objects cultivated in vitro. Micro-biological studies have shown that these are mainly such species as Brevibacillus sp., Moraxella sp., Alcaligenes, Bacillus spp., Brachybacterium, Brevibacterium, Brevundimonas, Corynebacterium, Enterobacter, Klebsiella, Kocuri, Methylobacterium, Micro-bacterium, Oceanobacillus, Ochrobactrum, Pantoea, Pseudomonas, Ralstonia, Staphylococcus, Tetrasphaer spp [10, 11].

In addition, the task of the sterile culture initiation stage includes not only obtaining sterile micro-plants, but also regenerated plants capable of further growth. Therefore, at this stage of the research, it was important to identify the optimal type of explants and a nutrient medium for initiating a sterile culture.

As a result of the experiments carried out 70 days after the introduction into an aseptic culture, it was found that the maximum number of viable initial explants in both studied grape varieties was obtained by isolating the meristematic apexes and planting them on a nutrient medium according to the prescription of Quorin Lepuvavr with the addition of 6-BAP at a concentration of 0.1 mg/l.

At the same time, the survival rate of both meristematic apexes and axillary buds on a nutrient medium according to the prescription of Quorin Lepuvavr was 50% versus 16.6% for explants planted on a nutrient medium according to the prescription of Murashige and Skoog (Table 1).

The main task of the multiplication stage in the technology of clonal micro-propagation is to obtain the maximum number of regenerated plants that do not have physiological abnormalities and are identical to the original mother plant, suitable for further micro-cutting and rhizogenesis. At this stage, the decisive role is played by the species and varietal characteristics of the culture, the method of introduction into the culture in vitro, the type of explant, its structure, origin, orientation on the nutrient medium, its composition and conditions of subculturing. [9]. Traditionally, during clonal
micro-propagation of grapes, micro-shoots are elongated in length and subsequently divided into micro-cuttings bearing axillary buds.

Table 1. The dynamics of the survival rate of grape varieties of interspecific origin of the Aleshenkin variety at the stage of introduction into a culture in vitro (%)

| Option                       | Duration of subculturing at the stage of introduction into culture | Meristematic apexes | Axillary buds | Micro-cuttings |
|------------------------------|---------------------------------------------------------------------|---------------------|---------------|---------------|
| MS (w/h) (control)           | 7 days                                                              | 66.6                | 16.6          | 16.6          |
| QL (6-BAP 0.1 mg/l)          | 14 days                                                             | 83.3                | 50.0          | 50.0          |
| Axillary buds                |                                                                     |                     |               |               |
| MS (w/h) (control)           | 70 days                                                             | 66.6                | 33.3          | 16.6          |
| QL (6-BAP 0.1 mg/l)          |                                                                     | 66.6                | 66.6          | 50.0          |
| Micro-cuttings               |                                                                     |                     |               |               |
| MS (w/h) (control)           | 100.0                                                               | 100.0               | 66.6          | 16.6          |
| QL (6-BAP 0.1 mg/l)          |                                                                     | 83.3                | 83.3          | 16.6          |
| Least significant difference | P < 0.05                | 0.61                | 0.99          | 1.32          |
| Least significant difference | P < 0.05                | 0.91                | 1.49          | 1.98          |
| Least significant difference | P < 0.05                | 1.63                | 2.66          | 3.55          |

At this stage of the research, it was important to assess the aftereffect of the type of explants used when introduced into the culture in vitro on the further replication of the studied grape varieties of interspecific origin. For this, two successive passages of regenerant plants were carried out on a nutrient medium according to the prescription of Murashige and Skoog (MS) with the addition of 6-BAP at a concentration of 0.1 mg/l, and the morphometric parameters of development were taken into account on the 20th and 40th days of subcultivation.

As expected, the multiplication coefficient of micro-plants increased with each passage, and the advantage of mericlones introduced into the culture by meristematic apexes remained.

On the 40th day of subculturing after the first passage in the Aleshenkin variety, the average length of shoots in micro-plants introduced into culture by meristematic apexes was 10.2 cm versus 2.4 – 4.5 cm in micro-plants introduced into culture by axillary buds and micro-cuttings, the average leaf surface area was 5.6 cm² versus 0.6 – 1.8 cm², the multiplication factor is 8.5 units versus 2.0 – 3.7 units.

At the second passage, the experimental plants showed the effect of spontaneous rhizogenesis against the background of elongation of micro-shoots, especially in micro-plants introduced into culture by micro-cuttings.

Micro-plants introduced into culture by axillary buds were lost due to latent bacterial infection. Which is one of the contraindications for the use of this type of explants, since the bacterial infection located in the conducting system of micro-plants is in a latent state and the infected specimens do not differ externally, and the fact of the appearance of a bacterial infection from the conducting system of micro-plants when transplanted onto fresh nutrient media is often noted [10, 19].

On the 40th day of subculturing, the advantage of micro-plants introduced into culture by meristematic apexes was also revealed, in which the average length of shoots was 11.3 cm versus 5.3 cm for micro-plants introduced into culture by micro-cuttings, the average leaf surface area was 4.5 cm² versus 1.9 cm², multiplication coefficient was 9.6 units versus 7.0 units. (Table 2).
Table 2. Dynamics of changes in morphometric indicators of the development of micro-plants of grapes of Aleshenkin variety of interspecific origin at two passages at the stage of multiplication

| Explant type when introduced into culture in vitro | 1st passage | 2nd passage | 20th day of subcultivation | 40th day of subcultivation |
|--------------------------------------------------|-------------|-------------|---------------------------|---------------------------|
| meristematic apexes                              | 1.3         | 2.4         | 10.2                      | 11.3                      |
| axillary buds                                    | 0.8         | 2.3         | 4.5                       | 5.3                       |
| micro-cuttings                                   | 1.5         | 2.4         | 1.0                       | 1.4                       |
| Average length of shoots, cm                     | 1.0         | 1.0         | 1.0                       | 1.4                       |
| Average number of shoots, pcs.                   | 0.7         | 0.6         | 5.6                       | 4.5                       |
| Average total leaf area, cm²                      | -           | 0.6         | -                         | -                         |
| Spontaneous rhizogenesis, %                      | -           | -           | -                         | -                         |
| Multiplication coefficient units                 | 1.0         | 3.2         | 8.5                       | 9.6                       |

4. Conclusions
1. In the case of clonal micro-propagation of grapes of interspecific origin of the Aleshenkin variety, the optimal type of explants for initiating a sterile culture are meristematic apexes with a height of 100 – 150 μm, which are advisable to be placed on a nutrient medium according to the prescription of Quorin Lepuavr (QL) with the addition of 6-BAP at a concentration of 0.1 mg/l.
2. At the stage of multiplication, in terms of the multiplication factor, regenerant plants introduced into the culture in vitro by meristematic apexes after the first passage were 2.3 times and after the second passage 1.4 times higher than micro-plants introduced into culture by micro-cutting. Latent bacterial infection was revealed in micro-plants introduced into culture with axillary buds at the second passage.

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