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Ecological and trophic determination of the ontogenesis *in vitro* plants of the genus *Betula*

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Abstract

A comprehensive assessment was conducted to identify the features of decontamination formation, morphogenesis of primary explants of birch genus plants and their regeneration at the stages of obtaining aseptic culture, multiplication and induction of rhizogenesis. According to the 'step-by-step' principle, the following were selected: the best age of explant donors and the conditions of their cultivation, namely, a depository with diffused lighting and fungicidal and bactericidal protection. The effectiveness of the use of Blanidas 300 as a decontaminant was substantiated. The peculiarities of the trophic determination of plant objects by artificial nutrient media with different compositions of mineral elements were established. The effect of phytotoxicity caused by excess nitrogen in the Morayshire and Skoog nutrient medium and excess sulphur in the Lloyd-McCone (WPM) artificial nutrient medium was established. The effect of pH on the availability of elements of mineral nutrition was revealed; the peculiarities of heterotrophic nutrition were investigated; the determinants at the stages of rhizogenesis and adaptation were identified; and the peculiarities of the ontogenesis of plant objects were determined. For effective decontamination and improvement of the regeneration process of primary explants, it is advisable to use one-year-old juvenile donors, grown under diffused lighting in depository conditions and with a system of compatible fungicidal and bactericidal measures. Based on experimental data, the expediency of the principle of using the main and unloading nutrient media is substantiated.

Key words

explant, decontamination, nutrient media, plant hormones, rhizogenesis

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Introduction

Climatic changes in recent decades – characterized by warming, increased greenhouse gas and dust emissions, reduced water availability for plants, and more torrential precipitation – have caused significant changes in both urban and forest ecosystems. At the same time, representatives of the birch genus are vulnerable to climate change due to the sensitivity of these plants to rising temperatures and summer drought (Rojo et al. 2021). The centre of origin and early evolution of the birch genus is Central-Eastern Asia (Welander 1993). The soils in this region were formed under the influence of the podzolic process by coniferous and deciduous species with a significant level of precipitation in the rain zones of the subtropics and tropics. This indicates a leaching-type soil with a moderate or low level of mineral elements and a relatively high content of organic residues, which contributes to the wide distribution of this species (Jonczak et al. 2020).

The birch genus from the *Betulaceae* family is mainly represented by deciduous trees, shrubs and bushes widely distributed in Eurasia and North America (Beck et al. 2016). It has important ecological and economic role as forest forming, pioneer, field protection, soil improvement, decorative species, etc. (Fedoniuk et al. 2023; Dubois et al. 2020; Álvarez-López et al. 2020). There are more than 100 natural species, hybrids and forms of the birch genus, which are widely used in landscaping, forestry, agroforestry, fixing of gully-ravine systems and protective plantings (Ashburner and McAllister 2013). Representatives of the birch genus are sensitive to pollution (Beck et al. 2016; Kolek et al. 2021), yet their high ecological plasticity and genotypic variability enable them to quickly acclimatize to changes in the surrounding natural environment (Oksanen 2021).

In recent decades, varieties have been created that can withstand significant anthropogenic loads and have high ornamental properties. These include *Jacquemontii* and *Royal Frost* (Ashburner and McAllister 2013; Wright 2017).

Royal Frost variety of *Betula pendula* Roth genus is a tree up to 9–12 m high, with a pyramidal crown with a moderate growth rate. *Betula pendula* 'Royal Frost' is a hybrid variety developed from the varieties 'Whitespire' and 'Crimson Frost' in the United States.

The variety is characterized by a strong, straight trunk with a spectacular white bark. In young plants, the bark is brown. The leaves are diamond-shaped, purple-burgundy and glossy on long petioles. In autumn, orange and red shades are added to the main colour.

Both open sunny and partially shaded areas are suitable for growth. However, the full colour of the leaves opens up in sunny places. Soil requirements are not high. Frost resistance is high, and wind resistance is moderate. It is used in the agroforestry plantations and decorative plantings, in particular, in the landscaping of parks, alleys and compositions.

Jacquemontii variety of *Betula utilis* D. Don genus is a tree with an egg-shaped crown 10–20 m high with white decorative bark that contrasts against the background of green leaves. *Betula utilis* 'Jacquemontii' is native to the Himalayas, particularly Nepal, Tibet and eastern China. For the first time, it was described by Édouard Spach in 1841. The variety tolerates short-term drought, and very rich soil resources are not required. It is planted in sunny sites. Signs are preserved by vegetative reproduction, that is, by grafting or microclonal propagation.

For their widespread implementation in urban greening, agroforestry, the study of trophic and ecological effects on plant ontogenesis and rapid vegetative reproduction is relevant. The study of these issues and rapid large-scale industrial reproduction is possible with the use of biotechnological methods.

In the ontogenesis process, future functions of individual cells, tissues, parts of organs, which form the functional structure of the organs, and the biological system of a whole organism are revealed. Age-related changes in organism parts are also superimposed on the determining factors (Becerra et al. 2004; Zhang et al. 2020). Isolation of explants from different parts of the donor plant organism and from plants of different ages will lead to excellent physiologically determined *in vitro* development programmes in regenerants.

Microclonal propagation is a type of asexual vegetative reproduction under special conditions (Kushnir and Sarnatska 2005; Singh 2015). It is based on the accelerated production of numerous genetically identical forms of the original plant using biotechnological methods (Gupta et al. 2020). This method is promising both for scientific (Bridgen et al. 2018; Matskevych 2020; Vítámvás et al. 2020; Brand and Lineberger 1992; Isah

2023) and for commercial needs, for example, in nurseries (Purohit et al. 2011; Corredoira and Costa 2021).

The promising scientific application of this method lies in the possibility of creating controlled factor-static conditions for the cultivation of plant objects (Matskevych et al. 2019). This allows researchers to minimize the impact of extraneous factors on the analysis of the factor under study (Kushnir and Sarnatska 2005). Scientists have been developing technologies for *in vitro* propagation of representatives of the genus birch for more than 50 years (Huhtinen and Yahyaoglu 1974; Meier-Dinkel 1992; Brand and Lineberger 1992; Iliev et al. 1998; Rathwell et al. 2016; Vítámvás et al. 2020; Xuening et al. 2021).

However, there are a number of problematic issues in microclonal propagation related to the composition of nutrient media, species, varietal characteristics, etc. (Abdalla et al. 2022; Ioannidis and Koropouli 2024). Improvement of these technologies and their adaptation to the requirements of the domestic consumer is an urgent scientific and commercial issue (Chornobrov and Tkachova 2021; Cardoso et al. 2018).

The purpose of the research was to investigate the complex of ecological and trophic determinants of the ontogenesis of representatives of the genus *Betula* for the optimization of the microclonal propagation protocol. To achieve this goal, the following tasks were set: (1) to obtain aseptic culture of *Jacquemontii* and *Royal Frost*; (2) to find out the peculiarities of the ontogenesis of plant objects under cultivation on different prescriptions of the nutrient medium; (3) to identify the effect of pH on the availability of mineral nutrition elements; (4) to investigate the peculiarities of heterotrophic nutrition; and (5) to establish determinants at the stages of rhizogenesis and adaptation.

Material and methods

Two forms of birch are involved in the study, which are among the most promising for urbanized landscapes and agroforestry plantations and belong to different botanical species of the birch genus: *Betula utilis* D. Don *var. Jacquemontii* and *Betula pendula* Roth *var. Royal Frost*.

In the first three stages, microclonal propagation was carried out under sterile conditions (Kulus and Tymoszuk 2024). Decontaminated signs were grown and stored in sterile conditions (in sterile dishes with a sterile environment).

Donors were selected from a certified source, under the conditions of constant monitoring of their state of health, genetic constancy and appropriate labelling (Fig. 1). We compared the rate of regeneration of birch plants *in vitro* from primary explants isolated from apical shoots of donor plants of different ages: 1 year, 3 years and 7 years.



Figure 1. Collection of mother plants (depository)

In order to increase the efficiency of decontamination of primary explants, the parent plants were treated with a fungicide (Previkur Energy 840 SL) and a bactericide ($Ag(NO_3)_2$) and an antibiotic (streptomycin) for 2–3 weeks. Shoots visually free from pests and diseases were selected for isolation. The selection was carried out at the beginning of bud awakening in a natural way.

Explants were cultivated in jars with a total volume of 200 ml with transparent polypropylene lids (diameter 66 mm). Jars were filled to 1/5 of the total volume with an artificial agarized nutrient medium (Fig. 2). Artificial lighting was provided at an intensity of 2400 lux. The photoperiod is 16 hours during the day. Cultivation temperature is 24±2°C.

Air sterilization was carried out with bactericidal lamps, in the laminar box – by filtration; nutrient medium and water for decontamination – by autoclaving, tools – in a sterilizer with an operating temperature of 250°C and more (Fig. 3).



Figure 2. Cultivation of birch plants *in vitro* in culture containers with a volume of 200 ml on light racks

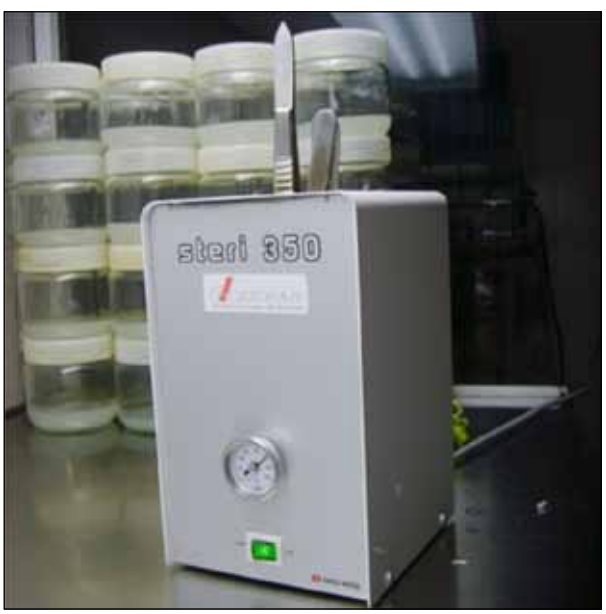


Figure 3. Sterilization of instruments

According to Kors (2010/2012), basic options of environment media (Fig. 4) were applied, which differed in the content of the mineral parts, that is, trophic complex (quantitatively and qualitatively in terms of the content of macro- and microelements). The remaining components of the media were the same depending on the stage of microclonal propagation, in particular, sucrose, vitamins and hormones.

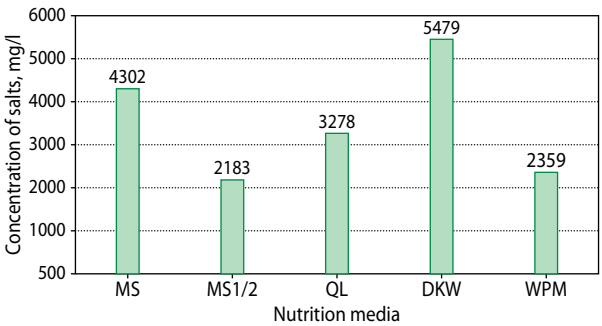


Figure 4. Concentration of salts in nutrient media

The repetition of research is fivefold in space and threefold in time. For one biological repetition, the average indicator for plant objects in the culture container was taken. The results obtained for the indicators were elaborated using the main statistical data parameters, which include the arithmetic mean and standard error. Statistical processing of experimental data was performed using the MS Excel analysis package.

For the phytohormonal determination of the root formation initiation process, the following combinations of substances, synthetic analogues of plant hormones, were added to the nutrient medium of all variants: cytokinins – benzylaminopurine (0.1 mg/l); auxins – indolylbutyric acid (1.0 mg/l). The research options are as follows:

1. WPM–WPM–WPM–WPM–WPM–WPM
2. WPM–WPM–WPM–WPM–MS1/2–WPM

A stem with leaves longer than 1 cm was considered a microshoot. The length of the root system was calculated by the longest root. A complete phase of development was considered when 3/4 (75%) of the objects reached the appropriate state of development.

The sequence of the series of studies was carried out according to the 'step-by-step' principle, that is, the best variant of the previous experiment was the control in the next experiment.

Results and discussion

The birch explant donors used in the experiments were grown in open ground conditions. The comparison rate of regeneration of birch plants *in vitro* from primary explants isolated from apical shoots of donor plants of different ages – 1 year, 3 years and 7 years – is shown in Table 1. Like other hardwood species, different birch species and genotypes respond differently to *in vitro* conditions and explant sources (Gaidamashvili and Benelli 2021). Most often, scientists recommend using WPM for birch (Lloyd and McCown 1980) or Murashige and Skoog (MS) (Murashige and Skoog 1962) environments. Explants were exposed to different nutrient media according to MS instructions.

Table 1. The influence of the age of donor plants on the features of decontamination and morphogenesis of primary birch explants in the MS medium

Index	Variety	Age, years		
		1	3	7
Decontamination efficiency, %	royal frost	31.4±4.09	23.2±3.11	14.1±2.07
	jacquemontii	19.5±1.99	11.4±2.01	7.4±1.86
Morphogenesis of explants, %	royal frost	11.9±2.02	9.0±1.98	6.1±2.02
	jacquemontii	6.3±0.98	5.1±0.88	3.3±1.07

As indicated by Abdalla et al. (2022), all processes of microclonal propagation are impossible without decontamination. This is because the components of nutrient media (elements of mineral nutrition, organic substances, etc.) are easily accessible not only to plant cells but also to bacteria and fungi (Abdalla et al. 2022; Chornobrov and Tkachova 2021). Not only pathogenic microorganisms can affect plant objects and 'spoil' the medium but also saprophytes, which are not directly harmful, can do so. However, changes in the composition of the medium, such as accumulation of toxic metabolites (e.g. fermentation products), can cause the death of plant objects. In the experiment, decontamination was carried out with a 20% solution of sodium hypochlorite for 20 minutes, followed by rinsing with autoclaved distillate. Sodium hypochlorite is a standard decontaminant used in microclonal propagation of a number of crops, including birch (Chornobrov et al. 2019; Neema et al. 2022).

We have established unequal indicators of the effectiveness of release from microbiological contaminants in explants from different age donors (1, 3 and 7 years). An increase in the age of primary explant donors decreased the efficiency of decontamination (Tab. 1).

In the *Royal Frost* variety, 14% of explants from 7-year-old donors were free from contaminants, and 23% from 3-year-old ones showed no signs of microbiological contamination. The highest efficiency of decontamination was in explants isolated from the youngest (1-year-old) explant donors – 31%.

A similar regularity regarding the influence of donor age on decontamination was established for *Jacquemontii* varieties. For all three variants of the age of the primary donors, the influence of the biological characteristics of the varieties was also established. *Royal Frost* had a higher percentage free from microbiological contamination than *Jacquemontii*. Research on other tree crops also established a correlation between the age of the donor plant and the ability of the explant to disinfection (Mohammed and Arkwazee 2024).

It is well known that the choice of explant and the appropriate disinfection protocol are key links in *in vitro* culture disinfection, reducing the negative impact of pollutants on plant tissues and promoting plant regeneration (Teixeira da Silva et al. 2016), because endophytic microorganisms in mother plants cause endogenous pollution (Gangopadhyay et al. 2017; Abdalla et al. 2022).

The older the explants, the more sensitive they are to disinfection (Teixeira da Silva et al. 2016). In our opinion, one of the reasons for the different indicators of the release of microorganisms from explants from donors of different ages is the unequal accumulation of endogenous microflora during the life period. Also, we assume that the difference between varieties in decontamination is related to the features of the covering tissues. In *Royal Frost* plants, compared to *Jacquemontii* plants, the surface of the leaf plates is less downy ('more glossy').

Thus, among the tested options for decontamination efficiency and morphogenicity was the option using one-year-old donor mother plants. This option of donors was used in subsequent studies.

We compared the efficiency of release from decontaminants and the number of morphogenic primary explants depending on the growing conditions of the donor plants (Tab. 2), namely, in open soil (field conditions) and depository (controlled factorostatic conditions of closed soil). Contaminant clearance efficiency for primary explants from *Royal Frost* depository-grown donors was 47% versus 30% for explants isolated from field-grown donors. For the *Jacquemontii* variety, these indicators were 39% and 21%, respectively.

Table 2. Influence of growing conditions of annual donor plants on features of decontamination and morphogenesis of primary birch explants in the MS medium

Index	Variety	Field conditions	Depository
Decontamination efficiency, %	royal frost	30.4±4.01	47.7±5.13
	jacquemontii	21.2±3.12	39.5±4.32
Morphogenesis of explants, %	royal frost	10.2±2.08	23.3±3.22
	jacquemontii	7.1±0.89	17.2±3.06

For both varieties, higher rates of morphogenic primary explants isolated from donors grown in depository conditions were obtained as well. We assume that this is due to a decrease in the accumulation of phenol-like substances under diffused lighting in donors from the depository and various accumulation of hormones. This coincides with our studies on other crops (Matskevich et al. 2019; Matskevich 2020). So, in subsequent studies, one-year-old donors growing in the depository were used.

In previous experiments, apical buds were used as such, which are most often used in microclonal propagation of crops (Welander 1993; Rathwell 2015; Covelo et al. 2018; Kumar et al. 1998; Amin and Jaiswal 1993). However, there are a number of studies on the effectiveness of meristems from different parts of the shoot (Hasegawa 1979; Tommasi and Scaramuzzi 2004; Gutiérrez-Nicolás et al. 2008). In the following experiment, we compared buds isolated from three zones of the shoot (Tab. 3): (1) apical (apex bud of the shoot), (2) medial (middle of a one-year-old shoot) and (3) basal (bottom of a one-year-old shoot). The highest indicators of both decontamination and the number of morphogenic explants were on the option using buds isolated from the medial part of the shoot as the primary explant.

The deviation in the indicators was within the margin of error. The option with the use of basal buds differed significantly from the others. On this option, the number of contaminant-free explants in the *Royal Frost* variety was 13±4%, and in the *Jacquemontii* variety, it was 9±4%, compared to 56±6% and 48±5% in the option with medial buds. Therefore, for further work, buds from the medial part of the shoot were used as primary explants.

Table 3. Influence of the origin of primary bud explants on the features of decontamination and morphogenesis

Index	Variety	Bud		
		apical	medial	basal
Decontamination efficiency, %	royal frost	49.8±5.46	56.7±6.32	13.5±3.98
	jacquemontii	38.3±4.05	48.5±5.23	9.1±2.86
Morphogenesis of explants, %	royal frost	20.3±3.11	31.3±4.21	6.4±1.88
	jacquemontii	16.6±3.16	27.6±3.09	3.21±2.01

In previous experiments, sodium hypochlorite (NaClO) was used as a decontaminating agent. However, our own experience and the research of other scientists testify to the shortcomings of NaClO as a decontaminant and the effective use of other substances (Covelo et al. 2018; Mamchur 2017).

We compared the effectiveness of using sodium hypochlorite and the following agents: $AgNO_3$, C_2H_5OH and Blanidas 300 (Tab. 4). Silver nitrate ($AgNO_3$) and ethanol (C_2H_5OH) were ineffective in removing both birch varieties from microbiological contaminants. However, according to literature data, they are effective for removing physical dirt and excess water (ethanol according to Kushnir and Sarnatska 2005; Matskevych 2020). Chornobrov and Tkacheva (2021) recommend using a stepwise method with treatment of *B. pendula* with 70% ethyl alcohol solution, 1.0% and 2.0% $AgNO_3$ solution, and 2.5% and 5.0% NaClO. A similar method of sterilization using 70% ethanol followed by 15% or 20% commercial bleach (Clorox®, The Clorox Company; 5.4% sodium hypochlorite) is suggested for *Betula lenta* L. (Rathwell et al. 2016).

The use of the Blanidas 300 drug exceeded sodium hypochlorite in terms of decontamination efficiency

and the number of morphogenic explants in both varieties. In particular, the efficiency of decontamination increased by 22% for the *Royal Frost* variety. The yield of morphogenic explants increased from 31% to 54%. A similar trend was noted in the *Jacquemontii* variety. Thus, the best decontaminant option is Blanidas 300.

Table 4. Decontamination and morphogenesis of primary birch explants with various antiseptics

Index	Variety	NaClO	$AgNO_3$	C_2H_5OH	Blanidas 300
Decontamination efficiency, %	royal frost	56±6	–	–	78±5
	jacquemontii	48±5	–	–	69±5
Morphogenesis of explants, %	royal frost	31±4	–	–	54±3
	jacquemontii	27±3	–	–	51±3

Our next task was to investigate the possibilities of trophic determination of ontogenesis at the stage of multiplication. According to Isah (2023), through the manipulation of the cultural medium, the determinants of morphogenesis can be identified and influenced.

In our previous experiments, the medium according to the recipe of MS was used. However, the development of regenerants was unstable after the third and subsequent graftings by the overlay method. In regenerants, the rosette of the shoots and the drying of the tops were noted (Fig. 5). Such symptoms are similar to excess nitrogen. According to the law of the maximum, an excess of this element of mineral nutrition suppresses the assimilation of calcium. It is the rosetteness and dying off of the tops that are a sign of insufficient calcium content or the complication of its assimilation (Matskevych et al. 2020). Therefore, in the next experiment in the MS medium, the amount of macronutrients, incl. and nitrogen-containing compounds were reduced by half, the rest of the medium components were left unchanged. The modified medium was hereafter referred to as MS1/2.

Comparing the morphogenesis of regenerants of Royal Frost and Jacquemontii varieties on the 30th day of cultivation on two types of media (MS and MS1/2) by such indicators as the height of regenerants and the reproduction coefficient (number of microshoots in the conglomerate), varietal features in changes in biometric indicators depending on the composition of the nutrient medium were established (Tab. 5).



Figure 5. Condition of explants at the fifth passage on MS medium, variety *Jacquemontii*

Table 5. Dynamics of morphogenesis of birch explants on different media

Index	Variety	Media				
		MS		MS1/2		
		age, years				
		1	5	1	5	7
Decontamination efficiency, %	royal frost	2.1±0.21	1.3±0.08	2.2±0.30	1.7±0.11	1.5±0.09
	jacquemontii	1.6±0.10	1.1± 0.06	1.7±0.11	1.5±0.08	1.5±0.10
Morphogenesis of explants, %	royal frost	39.5±3.09	12.1± 1.41	47±3.20	41.4±2.91	36.5±2.73
	jacquemontii	33.2±2.82	11.0± 1.22	38±2.73	32.6±2.43	27.5±2.64

When the number of cuttings was increased by the method of overlaying up to five, a significant difference was established between the height of the regenerants and the reproduction coefficient on media of different compositions of the mineral part. In the *Royal Frost* variety, the multiplication factor on the MS medium decreased by 38.1% during the fifth grafting and in the *Jacquemontii* variety by 31.2%.

After the fifth cuttings, further cultivation was technologically impractical. On the modified medium MS1/2,

the loss of morphogenic potential occurred more slowly (Fig. 6), so seven cuttings were studied. The coefficient of propagation of the *Royal Frost* variety decreased by 22.7% on the fifth and by 31.8% on the seventh cuttings.

An increase in the number of cuttings in both varieties led to a decrease in the height of the regenerants on the MS medium; when half the concentration of salts (MS1/2) was used, a tendency to decrease the size of the regenerants was observed. A significant decrease in the height of the plants was established after the fifth cuttings. Thus, despite the slower loss of morphogenicity of explants on MS1/2 medium, it cannot be recommended for long-term multiplication of these two birch varieties.

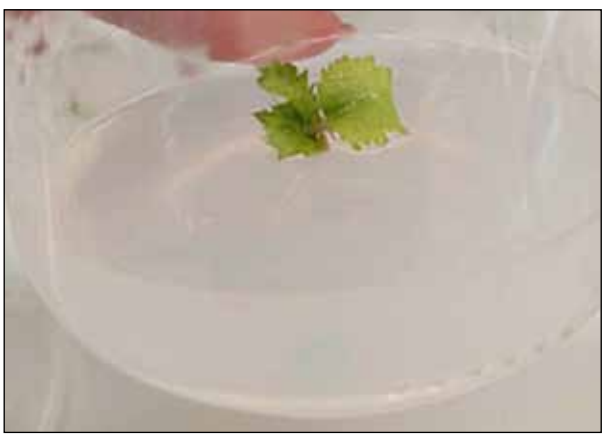


Figure 6. State of explants at the fifth passage on medium MS1/2, variety *Jacquemontii*

For wood species, the most commonly used media are MS, modified variants of MS, Hamburg's B5 medium and B5 modifications, Lloyd Makohen's medium (WPM) and Woody Kuniyuki's medium (DKW) (Phillips and Garda 2019).

The leading medium in the application for microclonal propagation of various species of birch is the classic MS medium (Zaki et al. 2011; Perez and Postigo 1989; Ide 1987; Chornobrov and Tkachova 2021). Nazari et al. (2024) recommend the combined use of MS and B5 media for the initiation of *in vitro* culture of birch *B. pendula* and *B. litwinowii*.

In the experiments of Lloyd and McCown (1980) with *B. lenta*, when investigating the effectiveness of DKW, MS and WPM basic media for the proliferation of shoots, a similar rate of propagation of shoots cultivated on MS and DKW media was established and the WPM medium was ineffective (Rathwell et al. 2016).

By contrast, some scientists recommend using WPM medium (together with MS or alone) for microclonal propagation of *B. platyphylla* and *B. papyrifera* (Magnusson et al. 2009), *B. oycoviensis* (Vítámvás et al. 2020), *B. litwinowii* (Payamnoor et al. 2024), *Betula medwediewii* R., *Betula megrelica* S. and *Betula raddeana* T. (Gaidamashvili and Benelli 2021).

Therefore, for further research at the multiplication stage, a comparison of morphogenesis was made on the following media: MS, MS1/2, DKW, QL and WPM.

The prescriptions of these media differ in the concentration of salts (Fig. 4). The highest concentration in the DKW prescription 5479 mg/l is the lowest in the WPM prescription 2359 mg/l. If we compare WPM and MS (4598/2359), the first is 1.95 times less concentrated. Also, WPM contains four times less ammonium nitrate than DKW and three times less than MS (Kors 2010/2012), to which regenerants of *Betula lenta* reacted negatively according to research data of Rathwell et al. (2016).

We found out a number of changes in morphological features in regenerants of *Royal Frost* and *Jacquemontii* as a reaction to trophic and phytohormonal determination of ontogenesis *in vitro*. As a result of such determination, morphological changes of the stem and leaves that make up the shoot can be observed. New internodes that are established during ontogenesis can also be formed in different ways. They consist of a part of a shoot and a leaf. A bud is formed in the axil of a leaf petiole. With the dominance of cytokinins in the nutrient medium, axillary buds form a conglomerate of microshoots.

We have established different reproduction coefficients, that is, the number of microshoots in the conglomerate, the height of the regenerants and the number of internodes of the largest microshoot in the conglomerate (Tab. 6), depending on the composition of the mineral part of the nutrient media. The research was conducted using the medium supplemented with 0.1 mg/l IMC and either 0.5 or 1.0 mg/l benzylaminopurine (BAP).

The option with the cultivation of explants on WPM medium was significantly different from the others in terms of the number of microshoots. In this option, the number of microshoots in the *Royal Frost* variety was

Table 6. Biometric indicators of birch regenerants on different nutrient media

Index	Cutting	Medium				
		MS	MS1/2	DKW	QL	WPM
Royal Frost						
The number of microshoots in the conglomerate, pcs	first	2.0±0.21	2.2±0.17	1.9±0.14	1.8±0.21	3.9±0.10
	fifth	1.3±0.18	1.7±0.31	1.8±0.30	1.8±0.33	4.1±0.22
Height of regenerants, mm	first	36.2±3.3	47.4±4.2	44.4±5.12	32.0±0.29	63.8±4.1
	fifth	31.1±2.90	41.1± 4.3	32.8±3.42	28.4±3.1	61.6±4.4
The number of internodes in the main shoot of regenerates, pcs	first	2.4±0.21	2.9±0.11	2.7±0.40	2.3±0.22	3.6±0.10
	fifth	1.7±0.18	2.3±0.16	1.3±0.31	2.1±0.19	3.5±0.08
Jacquemontii						
The number of microshoots in the conglomerate, pcs	first	1.4±0.22	1.7±0.19	2.0±0.11	2.2±0.21	3.7±0.29
	fifth	1.1±0.28	1.5±0.30	1.6±0.21	1.7±0.27	3.6±0.20
Height of regenerants, mm	first	32.3±3.1	38.6±5.5	41.2±3.3	30.4±3.1	59.9±3.6
	fifth	21.2±2.4	32.6±4.8	27.7±2.4	25.3±2.2	62.8±3.4
The number of internodes in the main shoot of regenerates, pcs	first	2.1±0.16	2.6±0.24	2.2±0.13	1.9±0.21	3.3±0.20
	fifth	1.3±0.15	1.8±0.19	1.1±0.15	1.8±0.22	3.4±0.18

3.9, and during the fifth cuttings, the tendency to increase (within the margin of error at ±0.2) up to 4.1 pcs. was observed in the *Jacquemontii* variety; this indicator remained almost unchanged (3.6–3.7 pcs.). In regenerants on other options of the nutrient medium, lower indicators of the number of microshoots and a tendency to decrease the reproduction coefficient, that is, shoots in a conglomerate that can be separated during cuttings, were noted.

Nutrients determine the course of metabolism and, as a result, the ontogenesis and morphogenesis of a plant object. Both the lack and excess of one or another element affect the availability and assimilation of other elements. That is, the deviation from the optimal amount affects the biometric indicators, in particular, the height of the shoot.

When comparing the height index of the regenerants on the 30th day of cultivation, it was established that the highest shoots were on the artificial nutrient medium according to WPM prescription. On this medium, the height was 63 and 61 mm in the first and fifth cuttings of regenerants of the *Royal Frost* variety and 59 and 62 mm in the *Jacquemontii* variety; the number of cuttings did not affect the height (the difference between the first and fifth cuttings was within the margin of error). On other options of nutrient media, shoots of regenerants significantly smaller in height were formed. There is also a tendency to decrease the height index from the first to the fifth cuttings on MS, MS1/2, DKW and QL media.

It should be noted that shoot height is a more quantitative feature. At the same time, the number of internodes characterizes both quantitative and qualitative changes, that is, plant development. The WPM medium was the most favourable for both the height of the shoots and the number of internodes (Tab. 6). Thus, the increase in the length of the shoot occurred not only due to the extension of the stem but also due to the formation of new organs.

Analysing the state of the issue regarding the effectiveness of WPM for the proliferation of the shoot with an increase in the number of cuttings (up to five), that is, the ability to support constant growth and development, we consider it necessary to pay attention to the concentration of salts and the history of the creation of prescriptions.

The DKW composition contains the highest concentration of salts. It was developed by Driver and Kuniyuki (1984) for the MKR of walnut (Tesliuk et al. 2022). The QL medium was developed for calciphiles, namely, drupe species (Kushnir and Sarnatska 2005; Murasige and Skuga 1962). WPM medium (Lloyd and

McCown 1980), suitable for tree species originating from nutrient-poor soils, has also been successfully used for blueberry propagation (Yavorska et al. 2016). In general, the concentration of salts (quantitative composition of media) is oriented to the evolutionarily formed features of plants, namely, the level of trophicity. The main niche for the growth of plants of the genus birch requires conditions similar to those found in Ukraine, namely, low content of mineral elements and often low pH (Bertelsen 2020). Therefore, from the point of view of metabolism and the experimental data obtained by us, the WPM environment is the best among the ones compared, which coincides with the results of other scientists' research.

It is known that plants evolved in certain ecological conditions differ in terms of the quantitative and qualitative content of mineral elements and soil acidity. This affected both the system of enzymes, which is genetically fixed, and the reaction to the concentration of H+ ions in the soil solution. During microclonal propagation, the ratio of biological plant species to the pH of the artificial nutrient medium is preserved, but there is a certain 'shift to the acidic side' (Kushnir and Sarnatska 2005). For example, almond, which grows well on soils with a pH of 7.0–8.0 in a nutrient medium, is inhibited at a pH of more than 6.3 (Matskevych et al. 2022).

We planted explants of *Royal Frost* and *Jacquemontii* varieties on options of the WPM nutrient me-



Figure 7. Development of birch regenerants under unfavourable nutrient medium acidity: pH 5.1, variety *Royal Frost;* pH 6.0, variety *Jacquemontii*



Figure 8. Development of birch regenerants at pH 5.5 nutrient medium acidity, varieties *Jacquemontii* (green in the centre) and *Royal Frost* (with burgundy colour)

dium, which differed in terms of acidity (pH): 5.1, 5.5 and 6.0.

It was stated that the option with pH 5.1 has signs of potassium, phosphorus and calcium assimilation deficiency, namely, purple spots on the lower leaves, no root formation, shortened shoot, dying of apical buds and signs of hyperhydration (Fig. 7).

The medium with a pH of 6.0 was characterized by the small shoot, and chlorosis caused by complications of iron assimilation was found on its apical part. The difficulty of assimilating Fe is characteristic of plants on soils with a high pH. Among the compared options, the optimal pH value was 5.5 (Fig. 8).

If we analyse the mineral composition of the WPM composition, almost half of it by physical weight is sulphur-containing compounds, namely, magnesium 180 mg/l and potassium 990 mg/l sulphates. In our opinion, this amount of sulphur as a mesoelement is excessive. As is known, the signs of an excess of sulphur are the gradual yellowing of leaf plates from the edges, their roughness, the edges turning inward and then browning and dying. Sometimes, the leaf plates acquire not a yellow shade but a lilac-brown shade.



Figure 9. Symptoms of excess sulphur in the nutrient medium, *Royal Frost* variety

The corresponding symptomatology was observed by us during long-term cultivation, that is, with 10 or more consecutive cuttings by the method of overlaying (Figs. 9, 10). The effect of the accumulation of phytotoxic excess hormones and nutrients was established in our previous studies (Matskevych et al. 2019; Chornobrov et al. 2023). One of the ways to combat 'fatigue' from long-term cultivation in the same medium is the use of a two-medium system: a main medium and an unloading medium with different composition (Gaidamashvili and Benelli 2021; Vítámvás et al. 2020). We used MS1/2 as the unloading medium.



Figure 10. Visual signs of deficiency caused by excess sulphur in the nutrient medium, variety *Jacquemontii*

The effectiveness of the use of combinations of 'basic and unloading media' was tested at the III stage of microclonal propagation – induction of rhizogenesis. The scientists studied the determinants of rhizogenesis: McClelland et al. (1990), Iliev et al. (2001), Vaičiukynė et al. (2017), Druege et al. (2019) and Chen et al. (2024). For the induction of rhizogenesis of woody crops, in particular, birch, WPM medium is mainly used (Chen et al. 2024; Vaičiukynė et al. 2017); therefore, in our experiments, this medium was also used as a basic medium with unloading MS1/2, since the low salt concentration in the nutrient medium improves rooting (Srivastava et al. 1985; Gaidamashvili and Benelli 2021).

For the phytohormonal determination of the root formation initiation process, the comparison was made according to the following indicators: the length of the root system, the number of roots, shoot height and the number of internodes (Tab. 7). With unchanged propagation on WPM, the length of the root system was 36 mm, and from the application of the unloading medium, the length of the root system of the *Royal Frost* variety increased to 69 mm and *Jacquemontii* variety to 11 and 37 mm, respectively. The number of roots

Table 7. The influence of combinations of nutrient media on the features of rhizogenesis of birch regenerants during long-term cultivation

Option	Index			
	the length of the root system, mm		number of roots, pcs.	
	...WPM–WPM...	...$MS_{1/2}$–WPM...	...WPM–WPM...	...$MS_{1/2}$–WPM...
Variety *Royal Frost*	36±3	69±4	2±1	7±2
Variety *Jacquemontii*	11±3	37±3	5±2	14±2

Table 8. The influence of combinations of nutrient media on shoot formation in birch regenerants during long-term cultivation

Option	Index			
	height of a shoot, mm		number of nodes, pcs.	
	...WPM–WPM...	...$MS_{1/2}$–WPM...	...WPM–WPM...	...$MS_{1/2}$–WPM...
Variety *Royal Frost*	36.3±2.21	75.5±3.84	4.1±1.59	9.1±1.99
Variety *Jacquemontii*	29.4±1.98	65.2±3.32	3.3±1.42	7.4±2.43

from the MS1/2–WPM combination also increased significantly: 3.5 times in the *Royal Frost* variety and 2.8 times in the *Jacquemontii* variety. The following regularity was established between the varieties in both options of the nutrient medium: the *Royal Frost* variety has a longer root system than the *Jacquemontii* variety. However, the variety *Jacquemontii* formed a root system with more roots.

The experiment of Gaidamashvili and Benelli (2021) with the endangered species *Betula medwediewii* R., *Betula megrelica* S. and *Betula raddeana* T. showed that the optimal medium for rooting shoots *in vitro* was WPM nutrient medium (with 1 μM IMC content), which prevailed over MS1/2 according to rooting efficiency. These results do not contradict our research since, first, we did not compare the effectiveness of two media, but we investigated their effectiveness when used together to optimize rhizogenesis *in vitro* during long-term cultivation on WPM medium. Secondly, it is necessary to take into account the peculiarities of the genotypes of different species, subspecies, forms and varieties of the birch genus (Iliev et al. 1998; Vaičiukynė et al. 2017; Zeps et al. 2022).

In our study, using different combinations of alternation of nutrient media also revealed differences in shoot morphogenesis (Tab. 8). In the *Royal Frost* variety, under unchanged cultivation only on the WPM medium, the height of the shoot was 36 mm, and in the case of the application of unloading medium (every sixth after WPM) MS1/2, the height of the shoot grew to 75 mm, that is, doubled. A similar pattern was established for the *Jacquemontii* variety.

The combination of main and unloading media also had a positive effect on the number of internodes in the shoot in both varieties, increasing it by 2 to 3 times.

Therefore, the alternation of WPM–WPM–WPM–WPM–MS1/2–WPM media during the propagation of *Royal Frost* and *Jacquemontii* birch had a positive effect on rhizogenesis and shoot formation in regenerants *in vitro* at the multiplication stage.

Post-aseptic adaptation is the final stage of microclonal propagation, which depends on whether the young plant will be able to overcome the stress during the transition *in vitro* – (*ex vitro*), *in vivo* (Dimitrova et al. 2021). According to McClelland et al. (1990), up to 50% of additional roots of *B. nigra* formed after transplantation *in vitro* either died immediately or were temporarily preserved during acclimatization without growing.

Each plant genotype requires an individual approach to substrate selection for adaptation (Chornobrov et al. 2023). A number of researchers suggest using substrates based on peat and perlite (Simola 1985; Chornobrov et al. 2023). Post-aseptic adaptation was carried out using peat and perlite substrate in the conditions of a normal wet chamber (illumination intensity 3000 lux, CO_2 content uncontrolled from 500 to 600 ppm) and a bioreactor (illumination intensity 11000 lux, CO_2 content controlled from 1800 ppm). Records were made on the 14th and 30th days of cultivation.

A significant difference in the increase in biometric indicators was established between the plants adapted in

Table 9. The influence of cultivation conditions on the adaptation of birch plants *in vitro* on the 14th and 30th days of cultivation

Adaptation conditions	Basis of the substrate	Shoot height, cm		Root length, cm	
		Royal Frost	*Jacquemontii*	*Royal Frost*	*Jacquemontii*
14th day of cultivation					
Wet chamber	peat	8.8±0.60	7.3±0.55	3.2±0.41	3.0±0.30
	perlite	9.7±0.72	7.8±0.62	3.6±0.49	3.0±0.42
Bioreactor	peat	20.1±1.91	17.6±1.31	19.3±1.65	13.4±1.17
	perlite	23.1±2.10	18.4±1.76	20.6±1.72	15.7±1.22
30th day of cultivation					
Wet chamber	peat	15.3±1.13	11.7±0.81	7.7±0.51	6.4±0.30
	perlite	16.1±1.33	12.9±1.15	12.6±1.12	9.8±0.72
Bioreactor	peat	39.6±2.31	32.1±2.32	29.7±2.33	23.3±1.93
	perlite	45.4±3.48	33.7±2.64	33.0±2.84	27.6±2.44

a humid chamber and those in a bioreactor (Tab. 9). The researchers have established the high efficiency of using a bioreactor for micropropagation through organogenesis or somatic embryogenesis and adaptation of plants in bioreactors (Ziv 1994; Hahn et al. 2000; Paek et al. 2005; Kozai et al. 2005; Nguyen et al. 2020; Trasar-Cepeda et al. 2023). According to the results of our research on the effectiveness of the wet chamber and the bioreactor for adaptation, it was established that the difference in the biometric indicators of *Jacquemontii* (*Betula utilis*) and *Royal Frost* (*Betula pendula*) plants reached more than two times or more in favour of the bioreactor. No difference was found in the influence of such factors as the base of the substrate and the biological characteristics of the variety. At the same time, Chornobrov et al. (2023) in research on the adaptation features of *Betula interspecies* hybrid plants *ex vitro* established high efficiency as the basis of an agroperlite substrate.

High growth rates and high photoassimilating activity require nutritional adjustments. In particular, a week after cultivation, visual signs of phosphorus and potassium deficiency were noted (Fig. 11). Therefore, we carried out feeding with a solution of KH_2PO_4 (970 mg/l). Seven days after feeding, the signs of deficiency disappeared completely from the upper leaves and only partially from the lower ones.

Chornobrov et al. (2023) indicated that the effectiveness of plant adaptation is determined by a complex of internal and external factors. Under the conditions of intensive synthesis of organic matter, we established the possibility of post-aseptic cuttings by rooting the tops. In particular, Fig. 12 shows the plants that were planted on 22 January 2024. The top of the shoot separated from them on 29 January 2024 took root as of 7 February 2024, and two new shoots grew from the top donor plant.



Figure 11. Condition of birch plants before (1, 2) and after feeding (3, 4), where 1, 3 – *Royal Frost* variety; 2, 4 – *Jacquemontii* variety

Therefore, this technique will contribute to an increase in the reproduction coefficient and yield of plants during microclonal propagation of *Jacquemontii* and *Royal Frost* varieties. This microclonal protocol of *Jacquemontii* and *Royal Frost* varieties will allow obtaining viable plants. The results of the research will become the basis for further studies on the determinants of the ontogenesis of plants of the birch genus in relation to the aggregate state of the media, the phytohormonal background at individual stages of microclonal propagation, cultivation conditions (lighting intensity, light composition, temperature regime), etc.

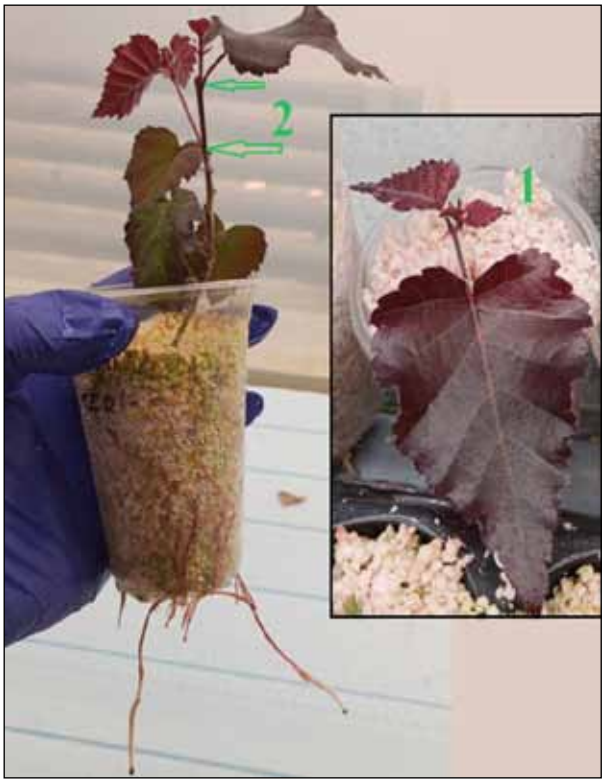


Figure 12. Post-aseptic cutting the top, variety *Royal Frost*: 1 – a plant transplanted from *in vitro* on 22 January 2024, from which the top was separated for rooting on 7 February 2024; 2 – two new shoots grow in the decapitation zone

Conclusions

In aseptic conditions *in vitro*, as a model closed artificial ecological system, the peculiarities of trophic determinants of the ontogenesis of explants of birch varieties belonging to two different botanical species (*Betula utilis* D. Don, *Betula pendula* Roth) of the birch genus were established. The conclusions were drawn, and recommendations were developed for use in industrial microclonal propagation of birch regarding the following elements of the technological process:

1. For effective decontamination and improvement of the regeneration process of primary explants, it is advisable to use juvenile one-year-old donors grown under diffused lighting (in depository conditions) and with a system of fungicidal and bactericidal measures.
2. Use Blanidas 300 as a decontaminant.
3. The best rhizogenesis and shoot development from the studied nutrient media were noted on the WPM medium. However, the effect of the accumulation of phytotoxicity during long-term cutting on the unchanged composition of the nutrient medium was established. In particular, symptoms of phytotoxic effect of excess sulphur on regenerants during long-term cultivation on WPM medium were revealed.
4. For long-term cutting, use WPM as the main medium, and MS1/2 as an unloading medium. Alternation of media from one discharge to five main ones.

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