In vitro conservation and propagation of Betula pendula Roth var. carelica (Mercklin) Hämet-Ahti

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Abstract. The article presents the results of clonal micropropagation of Karelian birch Betula pendula Roth var. carelica (Mercklin) Hämet-Ahti with the purpose of the valuable genotype conservation. We identified the best time for isolation of explants, at which the maximum viability of buds was noted, associated with a high content of auxins and cytokinins during this period of development. The optimal composition of the nutrient medium ensuring high regeneration of adventive shoots and their rooting has been determined. High survival rate of regenerants when transferred to ex vitro conditions has been achieved.

1. Introduction

Timber plantations are among the most valuable natural resources. Of particular interest is a variety of silver birch – Karelian birch Betula pendula Roth var. carelica (Mercklin) Hämet-Ahti. The wood of Karelian birch has a peculiar grain pattern, it is hard and highly decorative; for its marble-like texture, it has been named "patterned". Patterned wood is highly valued both in Russia and abroad. It is used for the production of expensive veneer for the furniture industry, as well as for artistic products. Karelian birch is a rare species and has a conservation status [1].

The natural forms of Karelian birch are usually dwarfed. As a result of many years of birch hybridization at the Forest Institute of the KSC RAS, V I Ermakov obtained specimens characterized by a beautiful grain texture and rapid growth. Of these hybrids, trees with a small tuberous type of trunk and a high-stemmed growth form are favoured, since they have the largest mass of patterned wood. The demand for patterned wood is increasing, but its procurement is limited due to the depletion of natural resources. With seed propagation, hybrids lose economically valuable traits that can be preserved only by vegetative propagation. Until now, vegetative propagation of the best Karelian birch hybrids has been problematic due to the low efficiency of propagation methods and high labor intensity of grafting. The use of valuable hybrids in plantations is limited for the same reasons.

In comparison with traditional methods of vegetative propagation, clonal micropropagation has a number of advantages: high reproduction rate; expansion of the seasonality of the work performed; the possibility of obtaining a large number of vegetative offspring of species that are difficult to reproduce under normal conditions or by using traditional methods; accelerating the transition of plants to the reproductive phase; the ability to work all year round and plan the release of plants by a certain date [2-4]. This method makes it possible to obtain homogeneous clonal offspring from the best adult trees with distinct economically valuable traits for industrial use [5, 6].
2. Methods and Materials
For clonal micropropagation of Karelian birch, the method of culture of tissues and plant organs was used [7].

The original material was the axillary buds of annual shoots of adult trees described below. Tree No 589 is a hybrid of the first generation of Karelian birch obtained by experimental crossing of the 35-40 year-old mother tree No 115 and the 30 year-old father tree No 24, both with a patterned wood texture. In the year of sampling, the age of the hybrid was 23 years. The growth form was high-stemmed, and the stem form was small-tuberous. The tree height was 7.5 m, and the diameter at a height of 1.3 m from the base of the trunk was 11.9 cm. Patterned wood grain was present throughout the trunk. Tree No 368 is a clone obtained by grafting cuttings from the mother tree No 5 (32 years old) with a patterned wood texture. The age of the grafted tree No 368 was 21 years. The growth form was high-stemmed, and the stem form was small-tuberous. The tree height was 6 m, and the diameter at a height of 1.3 m, 11.0 cm. Patterned wood grain was present above the grafting site (20 cm from the root collar) all over the trunk. Tree No 989 was a grafted tree of Karelian birch (ice birch); the tree age was 21 years.

The buds selected for the experiment were washed in a weak soap solution for 15 min, followed by rinsing with running tap water for 30 min. Further, the surface treatment of the planting material was carried out under aseptic conditions in a laminar box using 0.1% diacid, 3% hydrogen peroxide and 70% ethanol as sterilizing solutions. The exposure to sterilizing agents ranged from 0.5 to 10 minutes. Then the material was washed with sterile distilled water three times for 15 min.

The explants were placed on a nutrient medium in test tubes or flasks. The preparation and sterilization of culture media was carried out according to the existing recommendations [8-10]. For cultivation, the nutrient medium Murashige and Skoog (MS) [11] was used, modified in the composition and concentration of growth regulators of 6-benzylaminopurine (BAP), kinetin (Kin), 3-indoleacetic acid (IAA), and indolyl-3-butyric acid (IMA). Reproduction was carried out under controlled conditions with a 16-hour photoperiod, at a temperature of 24 °C and air humidity not less than 70%.

The regenerated plants were transplanted into containers with a soil substrate and kept in a growing room.

3. Results and Discussion
When developing a method for clonal micropropagation of birch, three stages of micropropagation described by N V Kataeva and R G Butenko [8] were implemented. Let us consider the cultivation of explants in stages.

3.1. Explant preparation
The vegetative buds of Karelian birch hybrids were isolated at different periods of the annual cycle: shoot growth (May, June, July), latent growth (August, September, October), and forced dormancy (February).

In our studies, we found the dependence of the morphogenetic activity of callus tissue on the physiological state of both the donor plant and the vegetative bud itself. We present characteristics of the morphogenetic activity of explants during the periods of active growth (May), latent growth (August - September), and forced dormancy (February). From the vegetative buds selected in May, after 7-10 days, a shoot with sufficiently developed leaves grew. When it reached 1.0-1.5 cm in length, it was cut off and transferred to the growth medium; at the base of the bud, a dense tuberous callus of yellow-brown color formed at the same time. Three weeks after planting, the callus doubled in mass. Small green meristematic foci appeared on its surface, from which regenerants developed. On one callus 1.5 x 1.5 cm in size, 29-41 shoots developed. This morphogenic callus was further used in experiments for plant regeneration. There was no need for additional subculturing on a nutrient
medium to stimulate bud formation. The grown shoots were transferred either to the rooting medium or used for multiplication.

Buds isolated in autumn (August - September) during the transition from vegetation to dormancy behaved differently *in vitro*. At the beginning of cultivation, a dense lumpy callus was also formed at the base of the bud. Shoots did not develop from the bud, and the integumentary scales gradually died off. In this experiment, the callus was subcultured twice. In the process, it increased in mass and turned green, but the meristematic foci did not form. The callus was transferred to a medium for the induction of bud formation, containing an increased concentration of BAP. To prevent the callus from drying out, a semi-liquid nutrient medium was prepared. Since high concentrations of phytohormones can alter the morphophysiological characteristics of the resulting plants, cultivation on this medium was short-term. After subculturing twice on a budding medium, the callus was transferred to a shoot growth medium. After two or three subcultures on this medium, the formation of a mass of small green shoots with miniature leaves on the upper and lower sides of the callus was observed. The grown shoots were then used for rooting or, if necessary, for multiplication of shoots.

In an experiment with explants isolated in February (a period of forced dormancy), a callus formed after 24 days. It was cultivated on a budding medium and after four weeks transferred to a shoot growth medium. Two months after cultivation, shoots began to form on the callus. These shoots were further used for rapid clonal micropropagation by activating axillary meristems and obtaining numerous lateral shoots.

Thus, testing of plant material at different times of the year revealed different regenerative capacity of callus. The lowest regenerative capacity was observed in the callus obtained from buds isolated in autumn during the transition from latent growth to dormancy; while a slight increase in activity was observed in the callus isolated during forced dormancy. The callus obtained from spring buds had the highest regenerative capacity. During this period, the maximum viability of the bud meristems, increased growth intensity and the highest yield of regenerated shoots were observed. Undoubtedly, this is due to the physiological state of a tree at the time when the explant is taken, with a change in the biochemical rhythms of metabolism of substances, including phytohormones, when the plant goes through periods of growth and dormancy.

We made an attempt to estimate the content of endogenous phytohormones in the vegetative buds of Karelian birch in the annual cycle of its development. Cytokinin substances, auxins (IAA) and abscisic acid (ABA) were determined by enzyme immunoassay. Their content fluctuates depending on the season.

In spring, buds break out, and an active growth of annual shoots starts. This period is associated with a high intensity of physiological processes, as well as with a high morphogenetic potency of vegetative buds. It is during this period that the highest content of auxins and cytokinins in the buds is observed. The interaction of cytokinins and auxins controls growth and differentiation of cells and tissues. In June, the IAA content drops sharply and remains at a low level until September. The content of cytokinins is still quite high in June, a decline is observed in July, and an increase, in August–September. In autumn, in a number of arboreal plants, the processes of formation and differentiation of buds take place, and auxins play an important role as stimulators of these processes. According to Chernobrovkina et al [11], from August to October the buds of Karelian birch grow vigorously, accumulate nutrients, and it is at this time that they are not able to break even under favorable conditions. In winter, the content of cytokinins is low, and the content of auxins drops sharply from December to March. In spring, before bud break, the content of these growth regulators increases.

According to literary sources, the level of ABA increases when plants are exposed to unfavorable environmental factors. It is this phytohormone that plays a decisive role in the regulation of the protective reactions of plant organisms in response to stress [12, 13].

The presence of natural inhibitors is associated with such phenomena as the dormancy of buds and other organs (for example, storage organs). According to our data, the content of abscisic acid in bud tissues is relatively low from spring to December; it was not found in May. The greatest content was
found from December to March, and this is consistent with the fact that at this time buds are in a state of forced dormancy.

Thus, the data show that the level of hormonal regulators in birch buds corresponds to the period of the plant's annual development cycle. It is obvious that the morphogenic activity of callus tissue obtained from buds isolated at different periods is associated with the level of phytohormones. Based on the data obtained, it becomes possible to regulate the concentrations of phytohormones (in particular, 6-BAP and IAA) added to the nutrient medium for in vitro cultivation of buds in accordance with the period of the annual development cycle in which explants from donor plants are isolated.

3.2. Selfmicropropagation

At the stage of selfmicropropagation, small pieces of green callus forming shoots served as material for the formation of a cluster of shoots. The shoots emerging on the callus grew for 4-5 weeks and, after reaching a length of 2.5-3.0 cm, were separated and used for multiplication, i.e., the grown shoots were pruned at the base and placed horizontally on a fresh medium with the same composition, after removing the apical bud. This process was repeated many times, and the maximum number of shoots per explant was reached (figure 1).

![Figure 1. Multiple shoot formation in Betula pendula Rothvar. carelica (Mercklin)Hämet-Ahti.](image)

Organogenic callus was cleared of necrotic areas and divided into pieces for further subculturing. This process was carried out with an interval of 3-4 weeks; in the case of an extension of the period to 6-7 weeks, the death of callus was observed due to competition for nutrients between the shoots and the callus itself.

One shoot was able to produce 4-5 new shoots growing from axillary buds during a four-week cycle. Each vessel contained five shoots at the start of each multiplication cycle; therefore it was possible to obtain 20–25 shoots from one vessel per month.

3.3. Rooting of shoots

The 2.0–3.0 cm long shoots were transferred to rooting medium. Two types of medium were tested: agar- and liquid-based, with support of shoots on filter bridges. It was experimentally revealed that root formation on a shoot grown on a liquid nutrient medium occurs after 7–10 days, and in experiments with an agar medium, roots appear after 15–20 days. In addition, the liquid medium is convenient in that there is no need to clean the roots from agar when transferring regenerants ex vitro, since the damage is inevitable during this procedure.

When shoots were transferred to the rooting medium, the shoot length was not important: even 1 cm long shoots were able to root. However, shoot length affects subsequent survival and
acclimatization. After 10–14 days, the plants developed 4–5 roots. The rooting success of test-tube plants was 95%.

4. Conclusion
We established the optimal conditions for in vitro cultivation of *Betula pendula* Roth var. *carelica* (Mercklin) Hämet-Ahti, with the purpose of conservation of this valuable genotype. The optimal time for isolation of explants is spring, when the maximum viability of the bud meristems is observed. It was shown that this is due to the high content of auxins and cytokinins during this period of development. Cultivation of axillary buds in vitro occurs by the type of formation of callus tissue on a nutrient medium of Murashige and Skoog with the addition of amino acids and growth regulators, followed by subculturing and elongation of shoots on a nutrient medium with a halved concentration of macro-salts and sucrose. The rooting of regenerated plants was carried out on a liquid nutrient medium. A scheme has been developed for the transfer of regenerated Karelian birch from aseptic culture to soil, at which the survival rate of 95% has been achieved.

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