ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

The Impact Of Genotype At The Stages Of Clonal Micro-Multiplication Of Different Species Of Birch When Scaling The Technology For Obtaining Planting Material Through In Vitro Culture.

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ABSTRACT

Birch is the main leaf forest-forming species in Russia and promising culture for forestry. The developed methodology for birch clonal micro-multiplication was used for large-scale in vitro cultivating of 10 Betula pubescens and B. pendula genotypes. The genotype had a substantial impact on the characteristics of in vitro culture at the stages of multiplication and rooting. The technique of acclimatization made it possible to obtain adapted birch plants with a very high frequency – to 90-95%. Plants were transplanted five times within three months to assess the impact of growth period length on the planting material yield and quality. Bedding of in vitro plants in greenhouse in the middle of May and June contributed to the maximum yield and height of seedlings by the end of vegetation season. The opportunity for obtaining birch planting material when growing with a high density (to 900 pcs./m^2), thus reducing the plant prime cost, is shown. About 10 thousand of birch seedlings were obtained at the end of vegetation season.

Keywords: birch, forest plantations, large-scale micro-multiplication, genotype, acclimatization, planting material.

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ISSN: 0975-8585

INTRODUCTION

The emphasis in forestry is put on coniferous species, but foliage species of moderate climate, particularly, birch, are also of economic importance. *Betula* species are considered main foliage species in boreal regions [1]. Birch is the main leaf forest-forming species in Russia. It occupies 15% of the area of wooded lands [2], its most widespread species are downy birch (*B. pubescens* Ehrh.) and silver birch (*B. pendula* Roth). The use of birchwood is wide and diverse, that's why it's very important commercial foliaceous species in Russia, as well as in North and East Europe [3].

Clonal micro-multiplication is an important method for producing planting materials for forest plantations [4], however, the use of such plants in forestry is still limited by relative high price and low survival rate during acclimatization [5]. The development of an efficient technology of acclimatization will make it possible to cut the prime cost, reduce the period of planting material production and improve its quality. In vitro birch cultivating began in the 1980s and it was mainly carried out in Finland [6]. Tissue culture researches in Russia were mainly carried out with karelian birch [7], however, there are no reports on the large-scale multiplication of a great number of genotypes.

The modern nursery-gardening of forestry crops is marked by an increase in the production of planting materials with closed root system. In Scandinavia where wood-growers started to work upon this issue in the end of the 1960s, the best part of planting material is cultivated in this manner: for example, the share of such seedlings in Finland makes up about 99% [8]. The size of containers for keeping seedlings is one of most important factors that determine the cost of seedling production and their cost [9].

The purpose of the research is assessment of genotype effect at the stage of multiplication, rooting and acclimatization of in vitro birch plants when scaling previously developed technology, as well as the impact of growth period length in the cells of planting cassettes of minimum capacity on the planting material yield and quality.

EXPERIMENTAL CONDITIONS

The following species and subspecies of birch were used in the research: *B.pubescens* (3 genotypes), *B.pendula* (3 genotypes), *B.pendula* var. *carelica* (3 genotypes), as well as *B.pubescens* × *B.pendula* (1 genotype) hybrid. Single-noded segments of sprouts 10-15 mm in length, which were cultivated in the medium, containing WPM salts [10], MS vitamins [11], 0.3 mg/l of 6-benzylaminopurine, by 18 explants in a 330 ml glass container, were used for *in vitro* multiplication. Transplantation into fresh medium was carried out in 4-6 weeks depending on genotype. Shoot apecies, which were planted on the WPM medium without growth regulators for 2-4 weeks by 30 explants in a 250 ml plastic container, were used for rooting. All culture media contained 30 g/l of sucrose and 9 g/l of agar. Culture media were sterilized by autoclaving (121°C, 20 minutes), and plant growth regulators and vitamins – by filtering (Millipore, 0.22 µm) and were added to media following autoclaving. Plants were cultivated at a temperature of 22-24°C and 16 h photoperiod.

In vitro plants acclimatization was carried out following the previously developed technique [12]. Enrooted plants were bedded out in a greenhouse into plastic cassettes with substrate, containing peat (Klasmann TS1) and perlite in proportion 3:1. The substrate prior to planting was treated with Fitolavin (10 ml/80 l of substrate). The cassettes with plants were placed to racks with capillary pads and covered with layers of spunbond and polyethylene film for 2 weeks (humidity 90-95%), then a layer of film was taken off and the cassettes were being kept under the layer of spunbond within 1 weak (humidity 70-75%). After removal of the spunbond cassette, plants were left in the greenhouse for a week, during which there were three dressings (2 g of Nutrisol/5 l of water for 1000 plants), and then they were transplanted into the field (till the mid of May – in plastic covers). The plants were bedded out for acclimatization within 3 months with 3-week intervals (the beginning of April, the end of April, May, June, the beginning of July). The plants were transplanted into cassettes with a plant growing density of 667 pcs/m^2 in case of early planting (beginning and end of April) and 900 pcs/m^2 - in case of later planting.

The multiplication coefficient and rooting frequency were evaluated at the end of cultivating period on the corresponding medium. The number of adapted plants was recorded in 4 weeks after transplanting to greenhouse (before transplantation on the field). The seedling yield and height were determined following the

end of growth period (in the middle-the end of September). The statistical processing of results was carried out using Statistica 6.1 (StatSoft, USA).

RESULTS

The duration of explant subcultivation period (at a week interval) was determined based on the moment of reaching particular criteria by in vitro culture. The following criteria were used: an increase in the merithallus length to a minimum of 10 mm (from 4 to 6 weeks) for multiplication, and rooting of at least 80% of sprouts (from 2 to 4 weeks) for rooting. Type and genotype had a substantial impact on the behaviour of *in vitro* birch plants (Table 1). Downy birch plants grew very slowly, karelian birch plants – faster, and there were both fast-growing plants (#4) and plants characterized by delayed growth (bb4b) among silver birch genotypes. The multiplication coefficient depended on the genotype most. The coefficient of the genotype bp3f1 of downy birch for 5 weeks was 7.9, while bp1b – only 4.3. The multiplication coefficient of the genotype of karelian birch tr1 for 4 weeks was 7.4, while other genotypes of this subspecies – about 5 for 5 weeks. Silver birch featured genotypes with the slowest and fastest multiplication: bb4b showed coefficient 4.1 for 6 weeks, while #4 – coefficient 7.7 for 4 weeks.

Table 1: The impact of genotype on multiplication and rooting of birch *in vitro*.

Species	Genotype	Multiplication		Rooting	
		Period, weeks	Coefficient	Period, weeks	Frequency, %
B.pubescens	bp3f1	5	7.9	2	99
	bp4a	6	6.1	2	100
	bp1b	6	4.3	2	96
B.pendula	bb31	6	5.9	4	85
	bb4b	6	4.1	2	91
	#4	4	7.7	2	98
B.pub.×B.pend.	66-150	5	5.1	3	85
B.pubescens var. carelica	ks06	5	4.8	2	91
	kb81	5	5.3	3	88
	tr1	4	7.4	4	84

Downy birch genotypes were characterized by the maximum speed and rooting frequency – 96-100% of sprouts rooted within 2 weeks. This said, rooting frequency didn't correlate to multiplication speed. The genotype #4 showed similar results among other genotypes, and the rooting frequency of the rest genotypes of silver birch (including karelian birch) didn't exceed 91%, notably, the plants of bb31 and tr1 genotypes took roots slower (within 4 weeks) than others, but they multiplied faster than others.

Table 2: The impact of genotype on plant acclimatization

Species	Genotype	Greenhouse planting		
		beginning of April	May	beginning of July
B.pubescens	bp3f1	93.4 ± 2.2 bc*	88.4 ± 1.4 c	-
	bp4a	94.8 ± 2.5 ab	88.2 ± 3.7 c	99.3 ± 0.5 a
	bp1b	85.7 ± 3.9 cd	94.2 ± 2.4 abc	-
B.pendula	bb31	60.8 ± 4.0 f	90.6 ± 2.6 bc	97.2 ± 1.1 a
	bb4b	97.5 ± 1.4 a	95.5 ± 2.6 a	-
	#4	85.0 ± 3.1 cde	96.0 ± 1.8 ab	99.5 ± 0.5 a
B.pub. × B.pend.	66-150	79.1 ± 3.6 de	68.3 ± 4.2 d	-
B.pubescens var. carelica	ks06	-	96.9 ± 1.5 a	74.3 ± 3.3 b
	kb81	71.7 ± 3.1 ef	88.0 ± 1.5 c	71.1 ± 3.3 b
	tr1	75.5 ± 3.7 def	94.8 ± 1.0 abc	-

mean ± standard error

* different letters show significance of difference $p<0.05$

The developed methodology of acclimatization generally proved effective when using on a large scale and contributed to a high survival rate of birch plants of different genotypes, which in some cases was nearly 100% (Table 2). Downy birch genotypes generally differed with steady survival without major deviations in

ISSN: 0975-8585

planting time – 85.7-99.3%. Substantial fluctuations in acclimatization at early planting were typical for silver birch plants. Lowe survival rate in greenhouse was observed in karelian birch and downy and silver birch hybrid.

Three genotypes of birch were sampled for more thorough study of the impact of planting times on acclimatization – by one genotype for downy, silver, and karelian birch (Figure 1). The level of acclimatization frequency dependence on planting time was different for different genotypes of birch. It was relatively stable and high for the downy birch genotype bp4a, and lower and non-stable – for the karelian birch genotype tr1.

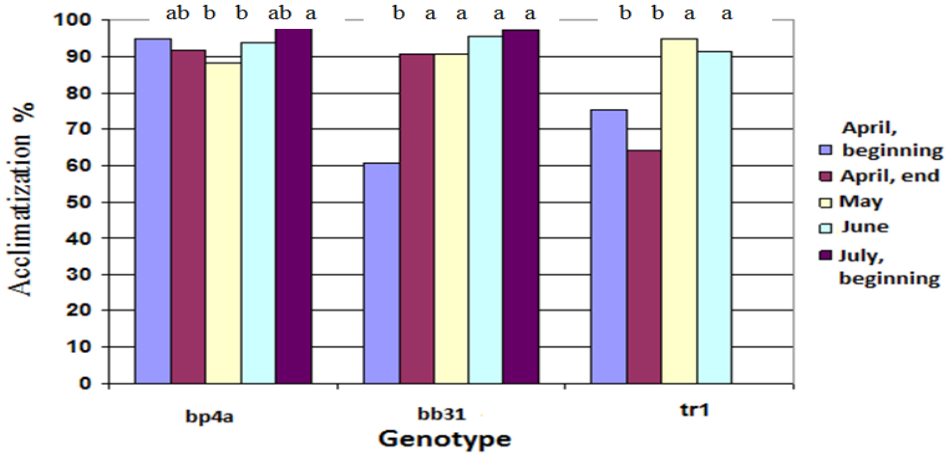

Figure 1: The impact of planting time on birch plant acclimatization

There was also the impact of genotype on the seedling yield upon the end of vegetation season, besides, most greatest fluctuations were evident when yielding plants in the beginning of April, when they were transplanted into the field in plastic covers (Table 3). The karelian birch seedling yield, as distinct from acclimatization frequency, was slightly different from the genotypes of other species of birch, but their height was minimum among all genotypes regardless of cultivation period. In general, downy birch plants were higher than silver birch plants, except genotype bb4b, which grew almost 2 times faster than other genotypes of this species (19.5-20.6 and 10.9-13.5 cm, correspondingly). The hybrid was intermediate in both parents by the rate of growth. Despite 6-week disproportion in the age, plants planted at the beginning of April and May were equally high, except genotypes kb81 and tr1 of karelian birch, which grew faster if they had been planted in May.

Table 3: The impact of genotype on seedling yield and height

Species	Genotype	Greenhouse planting					
		beginning of April		May		beginning of July	
		yield, %	Height, cm	yield, %	Height, cm	yield, %	Height, cm
B.pubes-cens	bp3f1	58.4±2.2 cd	15.0±0.8 b	69.6±6.3 bc	15.6±1.4 b	-	-
	bp4a	55.0±2.5 cd	14.8±1.1 b	77.8±3.0 ab	13.9±0.5 c	75.0±3.1 bc	9.6±0.2 a
	bp1b	81.1±4.2 a	12.4±0.6 bc	67.2±3.3 bc	14.0±0.5 c	-	-
B.pendula	bb31	47.8±2.5 d	10.9±0.3 c	82.1±5.1 ab	11.9±0.4 de	85.4±2.9 b	6.4±0.1 c
	bb4b	77.5±3.2 ab	20.6±0.1 a	79.5±4.4 ab	19.5±0.1 a	-	-
	#4	64.9±6.1 bc	13.4±1.6 bc	87.5±2.6 a	13.5±0.4 c	96.3±0.9 a	8.2±0.3 b
B.pub. × B.pendula	66-150	63.3±4.9 c	14.9±0.9 b	55.0±6.8 c	13.1±0.4 cd	-	-
B.pub. var. carelica	ks06	-	-	81.0±5.0 ab	9.1±0.3 f	71.4±3.3 cd	5.4±0.1 d
	kb81	66.1±5.0 bc	7.3±0.8 d	71.2±4.2 bc	13.5±0.1 c	70.4±3.2 cd	6.6±0.4 c
	tr1	62.1±2.6 c	7.8±0.3 d	76.3±2.2 ab	10.6±0.2 ed	-	-

Birch seedling yield was maximum at the planting of in vitro plants in greenhouse in June (81-88%), and to a lesser extent in May (76-82%) (Figure 2). The lowest seedling yield was observed at early planting – at the beginning and at the end of April (46-67%).

ISSN: 0975-8585

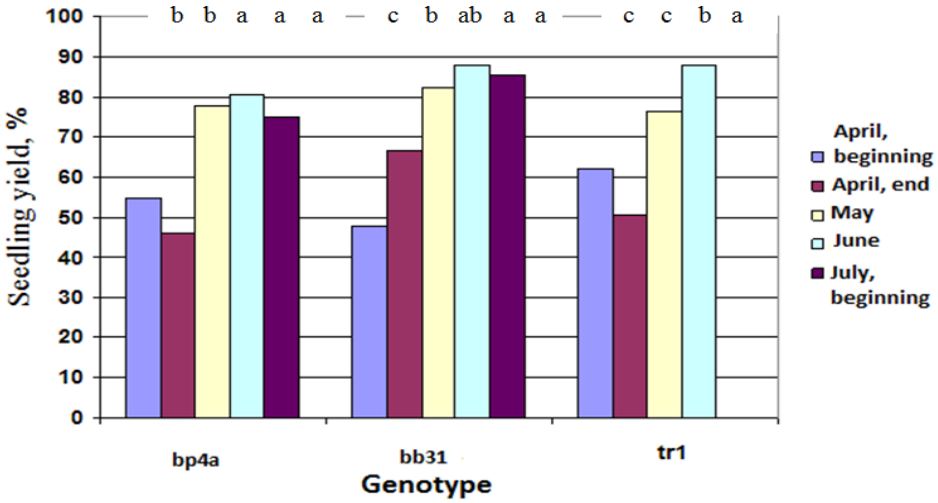

Figure 2: The impact of planting time on birch seedling yield

The planting time had a substantial impact on seedling height by the end of vegetation season (Figure 3). The height of plants of all genotypes was significantly lower than the height of youngest plants, which were planted in greenhouse at the beginning of July. The genotype bp4a had no significant changes in height for other terms of planting, while plants with genotypes bb31 and tr1 planted at the beginning of April were significantly lower.

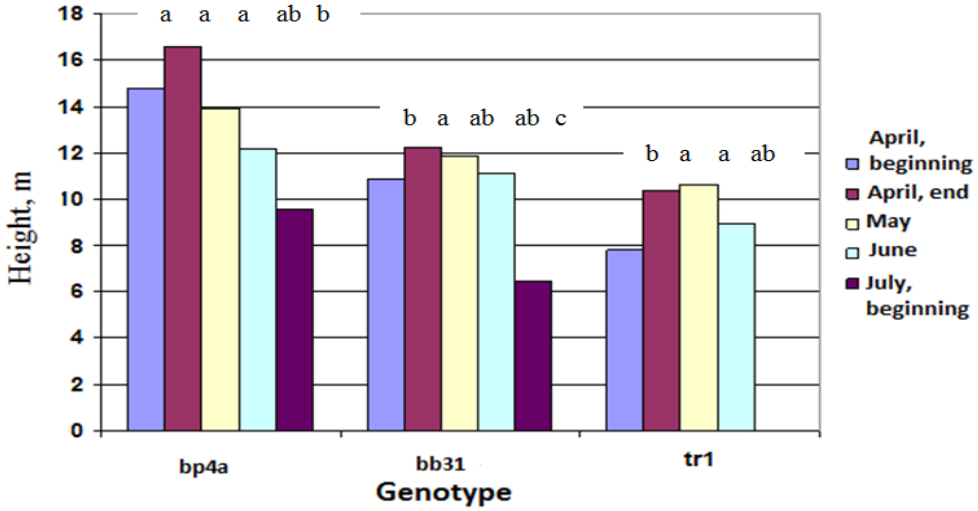

Figure 3: The impact of planting time on birch seedling height

DISCUSSION

An increase in the share of forest plantations, which yield is much higher than natural forests' is a differential characteristic of present-day forestry. For example, in 2000 forest plantations occupied only 3% of forest areas, however, they produced 27% of total wood [13], and, according to Food and Agricultural Organization, plantations will give to 64% of total industrial roundwood by 2050 [14]. High yielding capacity on plantations is partially achieved due to treatment, however, its primary reason is the use of high-yielding genotypes. However, traditional methods of multiplication fail to provide either genotype integrity (at propagation by seeds) or fast propagation for a limited time (vegetative multiplication). In this context biotechnological methods – clonal micro-multiplication – are increasingly important to this end.

As distinct from grass cultures, woody plants are much more complex objects for *in vitro* cultivating and it's reflected in the high cost of produced plants. It is known that genotype may have a substantial impact on the clonal *in vitro* micro-multiplication of woody plants. For example, significant differences were observed when cultivating *in vitro* clones of *Quercus robur* [15], *Acer saccharinum* [16], *Juglans major* × *J. regia* [17], as well as *Salix* species [18]. It may require optimization of culture conditions for a particular genotype, which will

contribute to an increase in the product cost. That's why it's important to choose such a methodology of *in vitro* cultivation, which would suit the most part of genotypes.

The previously developed methodology of birch clonal micro-multiplication proved sufficiently efficient and universal for a great number of genotypes relating to different species. Having said so, the genotypes of downy birch were marked by slow multiplication, but fast and high rooting, while the genotypes of karelian birch grew faster, but rooted slower and with lower frequency (Table 1). It's probably attributed to the hormonal status of these genotypes, that contributed to fast multiplication, but prevented rooting, and vice versa. The direct correlation between multiplication and rooting was identified for silver birch #4 genotype, which multiplied fast with a high coefficient, as well as rooted fast and became well established. It's important to note that differences between genotypes for providing desirable rates of multiplication and rooting required only an increase in cultivation time and not the change of culture media, which facilitates production process. The similar work was carried out with other wood species, for example, Preece et al. [16] proved applicability of the developed system of micro-multiplication for a wide range of *Acer saccharinum* L. genotypes. Fairly recently Palomo-Rios et al. [19] have developed an efficient method of willow micro-multiplication – medium 1/2 MS with 0.1 mg/l of indole butiric acid proved successful for 10 species and hybrids at each stage – induction of sprouts, elongation, multiplication, and rooting.

The success of clonal micro-multiplication at a commercial level is determined by availability of an efficient protocol of plant acclimatization, providing low cost and high rate of survival [20]. The acclimatization methodology we had developed proved effective on European ash micro-plants, where acclimatization frequency made up 96-100% [12]. A high rate of survival was also achieved when using the methodology on birch plants – to 95% and more (Table 2). The observed deviations mainly took place at spring transplanting (Fig. 1), most probably, because of fluctuations in temperature in greenhouse in this off-season. When adapting there were also significant differences between genotypes: karelian birch plants adapted worst, the genotypes of downy birch were marked by the highest stability.

CONCLUSIONS

The successful establishment of forest plantations depends on the use of quality planting materials, which are plants with closed root system. In this respect, the container capacity is one of most important factors, which determines the production cost and quality of seedlings [21]. The tendency for decreasing the planting container capacity is typical for present-day forestry: for example, seedlings of foliage species are being grown in Finland with a density of 150-400 pcs/m^2 [8]. In the framework of this research the birch plants were grown with a density to 900 pcs/m^2 and in case of transplanting in May and later, the seedling yield scarcely dropped below 70% (Table 2). In case of early bedding the low rate of survival of a number of genotypes was apparently caused by their high susceptibility to unfavourable weather conditions of external environment in the beginning-middle of May, when they were already on the field (under plastic covers). As distinct from acclimatization, the genotypes of karelian birch didn't differ from other genotypes of birch by seedling yield. The extended analysis of the planting time impact on seedling yield carried out on three genotypes of birch of different species also demonstrated the high mortality rate of plants transplanted in April (Figure 2).

The seedling size, which can be changed by the length of vegetation season through different planting time, is an important indicator of planting material quality. Generally, larger seedlings outgrew little ones in terms of growth rate and become better established, especially under stress conditions, for example, in case of drought [22]. Transplanting birch plants within 3 months with 3-week intervals contributed to identifying genotypic features: the genotypes of karelian birch were marked with the weakest growth, the genotypes of downy birch were most stable in respect to growth and the height of plants among the genotypes of silver birch differed two times (Table 3). The transplanting time had the most significant impact on the plant height: plants were considerably lower at later transplanting (July, beginning) almost for all genotypes, and to a lesser extent at transplanting in June (Fig. 3). The observations didn't show that early planting contributed to obtaining higher plants. This is the main difference of our research from the work of Close et al. [22], where it was demonstrated that eucalyptus seedlings planted a month earlier considerably exceeded more later ones in height and biomass, even if the container capacity was 2,5 times smaller. Apparently, low temperatures in May caused the stunt of plants of early planting time and it was impossible to overcome that stunt within summer months.

It is known that density of plant distribution, which influences plant morphology through light availability, is an important factor in forest nurseries [23]. For example, bedding density more influenced the larch seedling phenotype (112 or 224 pcs/m^2), than the increase of container capacity from 111 to 207 ml [24]. In this respect, in early planting times (April, beginning, end) plants were bedded out in cassettes with less frequent location of cells (667 pcs/m^2), than in the following months (900 pcs/m^2) in order to compensate in such a way the longer growth period, and, consequently, their greater foliage and lack of light (cell capacity was the same). The absence of substantial differences between beddings at the end of April and May proved that this approach is correct. The further increase in the survival ability and growth acceleration can be achieved by a number of techniques, for example, using growth-promoting substances that proved effective in European ash micro-multiplication [25].

We showed the opportunity for obtaining planting material of birch of different species and genotypes in mini-cells, which provide a high growing density and, consequently, lower seedling cost. All these technologies were used to produce planting material on a large scale – about 10 thousand of birch seedlings were cultivated at the end of vegetation season.

ACKNOWLEDGMENT

This research is supported by the Ministry of Education and Science of The Russian Federation (Project No. 14.607.21.0044 from 22.08.2014; unique identifier RFMEFI60714X0044).

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