In vitro selection of birch for tolerance to salinity stress

O S Mashkina¹,²*, T M Tabatskaya¹ and O M Korchagin¹

¹Department of Forest Genetics and Biotechnology, All-Russian Research Institute of Forest Genetics, Breeding and Biotechnology, 105 Lomonosova Street, 394087, Voronezh, Russian Federation
²Department of Genetics, Cytology and Bioengineering of the Faculty of Medicine and Biology, Voronezh State University, 1 Universitetskaya Square, 394018, Voronezh, Russian Federation

*E-mail: ilgis@lesgen.vrn.ru

Abstract. In vitro modelling of stress is one of the promising avenues for plant breeding for tolerance to negative environmental factors. In this study we examined the effect of NaCl (0.5%) on callusogenesis and morphogenesis of stem explants of different birch genotypes: Betula pendula Roth, B. pendula Roth var. carelica (Mercklín) Hämet-Ahti, B. pendula f. 'dalecarlica' (L.f.) Schneid., B. pubescens Ehrh. In our experiments we used pre-selected microclones from our in vitro collection on NaCl (0.2-1.0%) selective media. The clones were contrasted by the degree of their sensitivity to salinity (so-called 'stable' and 'sensitive' microclones). With the use of stem callus cultures we identified informative, simple and reproducible indicators for the selection of salt-tolerant genotypes. Among these indicators were the frequency of callus formation and the viability of callus cultures, which were significantly higher in 'stable' group of microclones. Polyploid birch clones (2n=4x=56, 2n=3x=42) were more resistant to salination compared to diploid clones (2n=28). Our study has shown that the selection of salt-tolerant birch lines can be based on the plants' genetic diversity presented in the collection (various species, varieties, hybrids, polyploids) and manifested in the process of in vitro cultivation, as well as in the cellular heterogeneity of callus cultures.

1. Introduction

In the context of global climate change, man-caused pollution, and the negative impact of environmental factors (drought, soil salination, etc.) on growth and productivity of plants (including forest trees), one of the urgent problems is to increase their resistance to abiotic stress factors [1,2]. The difficulty in dealing with this problem is the polygenic nature of the control of different traits (morphological, molecular, biochemical and physiological ones) that manifest themselves in response to abiotic stresses [3,4]. Therefore, selection for plant tolerance to stress abiotic factors is a rather complex, long and multi-stage process.

In vitro modelling of stress is one of the promising avenues for plant breeding for resistance to negative environmental factors [5-7]. The benefits of in vitro selection include a better control of the cultivation conditions and nutrient medium composition. The use of selective nutrient media (with increased levels of osmotically active substances, salts, herbicides, etc.) allows us to simulate natural stress conditions. This ensures the expression of resistance genes and helps us to select variants (individual cells, tissues, whole plants) with the desired characteristics [6], to preserve and propagated
them *in vitro* in the short term.

The most common abiotic stress factors are known to be drought and salination. *In vitro* culture has been used extensively to study the impact of these factors on plants [4,7]. Increasing concentrations of sodium chloride (NaCl) in the culture medium allows us to model both salinity stress and osmotic stress facilitating the selection of salt-tolerant and drought-resistant forms [6,8]. It was shown that NaCl salinity produce the greatest negative effect on plants [1,5,9].

There have been some positive results on selection of stress-tolerant plants through *in vitro* selection, but mainly for agricultural and fruit crops [4,6,8,10,11]. The researchers have used seedlings, callus tissues, cell suspension cultures, and isolated organs as explants [4,7,8]. For example, some scientists have used callus cultures generated through *in vitro* selection to obtain high-yield barley varieties resistant to drought and aluminium ions [11]; wheat lines resistant to drought and salination [12]; regenerative lavender lines tolerant to NaCl and low-temperature stress [13]. It has been noted that different plant species can require significantly different *in vitro* cell selection schemes [12,13]. This may be due to the fact that a selection scheme depends on the plant’s genotype, on the target trait, on the method to create *in vitro* selective model systems, on available information on the stress factor effect, on characteristics of callusogenesis and morphogenesis, and on other reasons [9,12].

Only few studies on *in vitro* selection have addressed forest tree plants so far [9,14-16]. For example, it has been demonstrated that different poplar and willow genotypes have significantly different degree of salinity resistance. That indicator was determined *in vitro* based on the growth rate and rhizogenesis of microshoots under saline conditions [9]. It also has been revealed that *Betula halophila*, the birch species endemic to China, has a high salt tolerance, which, according to the authors, is determined genetically and is related to the large number of genes expressed [17]. Also it was found that changes in the morpho-physiological characteristics of *Betula pendula* and *Betula pendula* Roth var. *carelica* (such as growth and rooting of shoots, the pigment content, etc.) are highly dependent on the concentration of cadmium in the nutrient medium [15].

Genetic diversity in plant species is a prerequisite for the perspective of successful breeding for stress tolerance. Another source of variability can be somaclonal variation (genetic and epigenetic), which arises during *in vitro* cultivation of cells and tissues (especially callus) and underlies *in vitro* cell selection [4,11,18].

Birch is one of the highly valuable forest-forming trees in Russia. It’s suitable for protective afforestation – activity that requires salt-tolerant and drought-resistant species. In the European part of Russia, the most common species are silver birch (*Betula pendula* Roth) and downy birch (*Betula pubescens* Ehrh.). A special place among various forms of silver birch is occupied by Karelian birch (*Betula pendula* Roth var. *carelica* (Mercrkin) Hämet-Ahti), because of its decorative patterned wood texture. Another interesting variety of silver birch is ‘dalecarlica’ birch (*Betula pendula* f. ‘dalecarlica’ (L.f.) Schneid.) widely used in landscape gardening due to its beautiful deeply dissected leaves.

The aim of our research was to study the effect of NaCl at the concentration of 0.5% on callusogenesis and morphogenesis of birch stem explants (a poorly studied object in this respect). Our primary goal was to develop a clear scheme for *in vitro* selection and to select salt-resistant regenerative lines. To do this, we applied a new approach – *in vitro* modelling of salinity stress with the use of two model systems: an *in vitro* collection of birch clones (which contains a large genetic diversity: species, varieties, hybrids, polyploids), and then – callus cultures obtained from microclones, opposed by the degree of their sensitivity to NaCl.

2. Methods and materials

This study was based on microplants of 8 birch clones: *Betula pendula* Roth (P1), *B. pendula* Roth var. *carelica* (Mercrkin) Hämet-Ahti (18k, Yu); *B. pendula* f. ‘dalecarlica’ (L.f.) Schneid (R2), *B. pubescens* Ehrh. (2psh, 3psh/1, 3psh/2, 6psh/3), opposed by the degree of their sensitivity to NaCl. We selected these birch clones from our *in vitro* collection through selective nutrient media
(NaCl 0.2-1.0%) and divided them into the ‘stable’ group (3psh/1, 3psh/2, 6psh/3, 18k) and ‘sensitive’ group (P1, Yu, R2, 2psh). The micro-shoots were 1-1.5 cm long and had one axillary bud [19]. All clones from the collection were maintained in vitro according to our method [20] by rare subculturing (once in 5-6 months) of microplants grown on ½ Murashige and Skoog (MS) hormone-free nutrient medium [21].

To obtain callus cultures from birch microclones, opposed by the degree of their sensitivity to NaCl, we used 1 cm long stem segments (internodes) as explants. Callus induction and proliferation took place on MS nutrient medium (18), supplemented with 6-benzylaminopurine (6-BAP, 0.5 mg/l) and α-naphthalene acetic acid (NAA, 2 mg/l). Morphogenic calluses were grown on MS medium supplemented by 6-BAP (1 mg/l), shoot regeneration in morphogenic callus took place on ½ MS medium supplemented by 6-BAP (0.5 mg/l) and indole-3-acetic acid (IAA, 0.2 mg/l).

The cultivation conditions were as follows: temperature of 25±2 °C, photoperiod consisting of 16 hours of light and 8 hours of darkness, illumination of 2.0 klx.

The analysis of primary calluses involved two variables: the frequency of callusogenesis (CF, %) – the ratio of explants that formed callus to the total number of explants; and viability of callus cultures (CCV, %) – the ratio of callus cultures that survived after 30 days of cultivation to the number of explants that formed callus (figure 1-2).

Living callus tissue is easy to distinguish from non-viable tissue: the viable one is light in colour, while dark brown spots indicate the progression of necrosis (figure 2). Previously, we had confirmed that this indicator can be used for pine tree. Using cytological analysis, we found that the dark brown cells did not contain nuclei, while in living tissue large nuclei were found in the middle of the cells [22].

The primary callus tissue was isolated and transferred to fresh media 30 days after the beginning of cultivation. The callus tissue was subcultivated every 30 days. The experiments involved both primary and recultivated birch callus of 2-3 passages. To obtain the primary callus, we placed 5-7 stem explants in one flask. In the experimental group (with salt exposure) we used 2-3 calli (primary or recultivated) for each culture vessel (flask or test tube).

The composition of the nutrient medium was modified by addition of NaCl at concentrations of 0.5%. That was done at the stages of formation and proliferation of the primary callus and regeneration of shoots in the morphogenic callus to create the provocative background. This concentration allowed us to identify the difference in the response of birch clones to salinity stress and provided a survival rate of 50% [19].
As a control, we used media without NaCl.

After salt exposure, birch callus cultures were recultivated 2-3 times under normal cultivation conditions (without stress) and then transferred to the morphogenic medium MS + BAP (1 mg/l). At the stage of shoot regeneration in morphogenic callus, we tested a ½ MS + 6-BAP (0.5 mg/l) + IAA (0.2 mg/l) medium with and without NaCl (0.5%). After 30 days of cultivation, we estimated the frequency of shoot regeneration (%). It was determined as the ratio of the number of calluses with shoots to the total number of morphogenic calluses.

![Figure 2. Callus tissue viability: viable tissue (on the left) and tissue with signs of necrosis shown by arrows (on the right).](image)

Each experiment was replicated 3 times. We used at least 20 callus cultures for each clone.

Shoots obtained in callus culture continued to grow and propagate on hormone-free ½ MS medium, supplemented with activated charcoal (2%).

The statistical analysis of the results was done using the ‘Stadia’ v.7.0 software (http://top-torrent.ws/soft-torrent/4463-camtasia-studio-70.html, ‘TechSmith Corporation’, USA). We used the Student's t-test to compare the samples.

3. Results and discussion

Previously, we developed a biotest system based on Scots pine callus cultures under saline conditions (on NaCl supplemented nutrient media) and demonstrated its potential for the selection of drought-resistant genotypes [22]. This became the basis for in vitro selection of deciduous woody plants (birch, in particular).

At the first stage of our research, we examined the salinity stress effect in vitro culture of microshoots of 10 birch clones [19]. We used genetically diverse birch material from our in vitro clone collection in the research [20]. Our study revealed that the genotype had a significant impact on the preservation of the explants and their ability to regenerate under salinity stress. We demonstrated that the gradual method of in vitro selection with a step-by-step increase in NaCl concentration (from 0.2% to 1.0%) is the best to differentiate the studied clones by their salt tolerance. The cultivation cycle used was as follows: 20 days of salt exposure, then 30 days of cultivation on medium without NaCl. After five repetitions of this cycle we differentiated the clones into ‘stable’ and ‘sensitive’. Stress tolerance was assessed based on preservation (viability) of cultures, their regenerative potential and morphological changes [20]. The preservation rate of ‘stable’ clones was higher (from 40 to 90% depending on genotype), their microshoots also had higher regenerative potential (50-80% of explants showed signs of shoot formation, there were 60-90% of rooted microplants). In the other group (so-called ‘sensitive’) these characteristics did not exceed 10-30%, 40% and 40-50%, respectively [20].

To confirm this differentiation and increase the efficiency of in vitro selection, we continued our studies using callus cultures. In this study we examine the effect of NaCl (0.5%) salinity stress on callusogenesis and morphogenesis of previously selected microclones, opposed by the degree of their...
sensitivity to NaCl (so-called ‘stable’ and ‘sensitive’). To select stable callus lines, selective agents are most often introduced into the medium at the stages of proliferation (when cells are actively growing and dividing) and morphogenesis of callus tissue [4,11]. Unlike researchers in previous studies, we additionally evaluated the primary callus-forming reaction of stem explants (fragments of micro-plant stem) in response to the increased concentration of NaCl in the culture medium. This was due to the fact that the ability of isolated tissue (primary explants) to form callus depends primarily on the genotype of the parent plant, and then on the cultivation conditions [18,23].

Our study demonstrated that primary birch callus cultures responded to salt exposure differently, the response depended on the clone (table 1). The frequency of CF and CCV were significantly higher in the group of ‘stable’ clone both in the control group (without salt exposure) and in the experimental group (with 0.5% NaCl).

We found that the induction of callusogenesis for both ‘stable’ and ‘sensitive’ groups of clones was limited by salt exposure (NaCl, 0.5%). Moreover, under salinity stress conditions, the interclonal differences were more pronounced. The ratio between the average values of CF in ‘stable’ and ‘sensitive’ groups of clones was 1.9 (45.1% / 23.9%) in experiment and 1.1 (and 70.4% / 61.1%) in control. The ratio between the average values of CCV in ‘stable’ and ‘sensitive’ groups of clones was 2.6 (47.6%/18.5%) in experiment and 1.4 (80.8%/57.6%) in control (table 1). As we can see, the ratio in the experiment was almost 2 times higher than in the control.

Callus formation as a reaction to salinity stress was particularly pronounced in the ‘sensitive’ group of clones. In our experiment CF and CCV for the ‘sensitive’ group ranged from 9.0% to 40% and from 10.1% to 27.2% respectively (versus 40-55.3% and 40-60.2% in the ‘stable’ group).

Thus, the birch clones previously differentiated in terms of their salinity tolerance (‘stable’ and ‘sensitive’) confirmed their status after their callus cultures were exposed to saline stress. We found that the frequency of callusogenesis and the viability of callus cultures are informative, reproducible, and relatively simple indicators, which can be detected visually. They can be used as evaluation criteria for the clones’ stress resistance under \textit{in vitro} salt exposure.

**Table 1.** Efficiency of callus formation in birch clones opposed by the degree of their sensitivity, grown on nutrient medium MS + NAA (2 mg/l) + BAP (0.5 mg/l) with NaCl (0.5%).

| Group   | Clone | Control, % | Experiment, % |
|---------|-------|------------|---------------|
|         |       | frequency  | viability     | frequency  | viability     |
|         |       |            |               |            |               |
| ‘stable’| 6 psh | 80.0±0.3   | 80.3±0.2      | 40.0±1.3   | 40.0±1.1      |
|         | 3 psh/1| 65.0±1.1   | 75.0±1.1      | 55.3±0.1   | 60.2±1.4      |
|         | 3 psh/2| 69.8±1.1   | 87.9±0.3      | 45.1±0.2   | 50.2±0.8      |
|         | 18k   | 66.6±0.8   | 80.0±1.3      | 40.0±0.4   | 40.0±1.1      |
|         | mean value | 70.4±1.3   | 80.8±1.1      | 45.1±1.4\textsuperscript{1} | 47.6±1.9\textsuperscript{2} |
| ‘sensitive’| 2 psh | 60.0±1.3   | 40.0±0.2      | 40.0±1.7   | 27.2±0.6      |
|         | P1    | 54.5±1.2   | 60.0±0.4      | 16.6±0.8   | 10.1±0.4      |
|         | Yu    | 70.1±1.3   | 60.2±0.2      | 29.9±2.5   | 20.0±0.3      |
|         | R2    | 60.0±1.1   | 70.1±0.2      | 9.0±0.6    | 16.6±0.7      |
|         | mean value | 61.1±1.3\textsuperscript{***} | 57.6±2.5\textsuperscript{****} | 23.9±2.7\textsuperscript{***,1} | 18.5±1.4\textsuperscript{****,2} |

\textsuperscript{***}The differences between the ‘stable’ group and the ‘sensitive’ group are statistically significant at \(p<0.001\); \textsuperscript{1}the differences between the experimental and control groups in CF are statistically significant at \(p<0.001\); \textsuperscript{2}the differences between the experimental and control groups in CCV are statistically significant at \(p<0.001\).

In both birch groups, calluses showed heterogeneity in structure (dense, globular, or loose) and in colour (yellow, white, light green, or grey) (figure 3). The negative effect of sodium chloride on callus
formation was reflected in the inhibition of the growth of the primary callus and the appearance of necrotic symptoms. The symptoms were detected visually and were seen as focal or diffuse lesion of the callus tissue. Areas of dark brown necrotic tissue stood out against the yellow or light yellow background (figure 3).

Figure 3. Types of callus tissue different in structure, colour, and viability after NaCl salt exposure (0.5%). The foci of necrotic callus tissue are shown by arrows.

In our experiment, the most viable and morphogenic calluses were green, dense and had globular structure. This is consistent with literature data [4,24]. Therefore, the selection of green dense callus cultures without signs of necrotic tissue helps to obtain morphogenic birch cultures. The importance of callus structure and colour should be taken into account not least because of literature data, according to which a number of plants could have different types of morphogenic callus, even loose or watery [4,24,25] – i.e., the types that are non-morphogenic in birch.

To obtain morphogenic callus, we recultivated it 1-3 times (1-3 passages) on MS + BAP medium (1 mg/l) without NaCl. All clones had 3 types of callus with different regeneration potential (figure 4): 1 – callus with multiple rhizogenesis (not capable of shoot formation in future); 2 – dense green callus (good for regeneration in future); 3 – light yellow callus with areas of dense green and white tissue. The light yellow and white calluses had low regeneration activity and viability.

The birch stem callus showed sharp interclonal differences in the morphogenesis. The first and second types of callus prevailed in ‘stable’ clones, the second and third types prevailed in the ‘sensitive’ ones.

Figure 4. Morphogenic reactions in callus cultures after salt exposure (NaCl, 0.5%): callus with multiple rhizogenesis (a); dense green morphogenic callus (b); heterogeneous callus in terms of consistency and colour (c).

One of important problems of in vitro selection is to preserve the regenerative ability of callus lines (or cell lines) selected on a selective background [6,12,13]. To regenerate the shoots, we selected portions of green dense morphogenic callus (1-3 passages) and transferred them to a ½ MS + 6-BAP
(0.5 mg/l) + IAA (0.2 mg/l), one part of which was supplemented with 0.5% NaCl (selective medium) and the other part remained without NaCl. When the calluses were exposed to sodium chloride at the stage of shoot regeneration, both group of clones (‘stable’ and ‘sensitive’) did not form shoots. This may be due to the previous salt stress and its prolonged inhibitory effect on in vitro morphogenesis. The data provided by literature shows that salinization significantly inhibits the ability of cells and tissues cultivated in vitro to differentiate and undergo morphogenesis [12,26].

With this in mind, we conducted the further research of shoot regeneration in birch callus in non-selective conditions (table 2). The results of the research showed that the frequency of shoot regeneration in the ‘stable’ group of clones was 4.6 times higher than in ‘sensitive’ group (on average, 21.3% versus 4.6%) (table 2).

| Group | Clone | The frequency of shoot regeneration, %
|-------|-------|-------------------------------------------------
|       |       | ½ MS + 6-BAP (0.5 mg/l) + IAA (0.2 mg/l)        |
|       |       | 1 passage | 2 passage | 3 passage |
| ‘stable’ | 6 psh | 16.6 | 0 | 11.7 |
|         | 3 psh/1 | 27.2 | 10.0 | 0 |
|         | 3 psh/2 | 21.4 | 16.6 | 11.1 |
|         | 18k | 20.0 | 11.8 | 0.0 |
|         | mean value | 21.3±1.0l | 9.6±1.5 | 5.7±1.5 |
| ‘sensitive’ | 2psh | 11.8 | 0 | 0 |
|         | P1 | 6.6 | 0.0 | 0 |
|         | Yu | 0.0 | 0.0 | 8.3 |
|         | R2 | 0.0 | 11.1 | 13.3 |
|         | mean value | 4.6±1.3*** | 2.8±1.2** | 5.4±1.5 |

Obtaining and passage of morphogenic callus was carried out on MS medium + BAP 1 mg/l; the differences between the ‘stable’ and ‘sensitive’ groups of clones were significant at: ***p<0.001 **p<0.01 *p<0.05; differences with 1 passage in the ‘sensitive’ group of clones are significant at p<0.001

All 4 ‘stable’ clones formed shoots, while in ‘sensitive’ group only two clones (2ps and P1) were able to form them (figures 5-6).

We found that the regenerative activity of the selected morphogenic callus also depended on the number of passages on the hormonal ½ MS + 6-BAP (0.5 mg/l) + IAA (0.2 mg/l) medium. When the number of passages increased from 1 to 3, the frequency of shoot regeneration in the ‘stable’ group of clones decreased almost 4 times (from 21.3 to 5.7%). This indicates that it may be appropriate to use morphogenic callus of the 1st passage, which in the ‘stable’ group had higher ability to form shoot (16.6-27.2% versus 0-16.8% for the 2nd passage) (table 2).

The regenerative birch lines obtained from callus were initially distinguished by various phenotypic abnormalities. They manifested themselves in inhibition of the shoot growth in height, a decrease in leaves size, their underdevelopment, and weak rhizogenesis. Shoots of ‘sensitive’ lines also showed signs of marginal necrosis in the leaves, there was a noticeable defoliation. However, in later subcultures, most of these negative changes disappeared (figure 7), which may be due to their non-hereditary (modificational) nature.
Figure 5. Different regenerative activity of callus cultures in ‘sensitive’ 2psh birch clone (on the left) and ‘stable’ 3psh/1 birch clone (on the right) on the 30th day of cultivation on ½ MS + 6-BAP (0.5 mg/l) + IAA (0.2 mg/l) medium. The callus of 2psh clone (on the left) do not regenerate shoots and is less viable (there are signs of necrotic tissue).

Figure 6. Regeneration of shoots in the stem callus after salt exposure (NaCl, 0.5%) in the salt-tolerant lines: 3psh/1s (a) and 18k/s (b, on the left) and absence of shoot regeneration in the ‘sensitive’ line P1 (b, on the right).

Figure 7. Restoration of growth and normal morphotype of regenerants obtained in vitro from birch callus cultures (6psh/s clone) in the process of gradual subcultivation (from left to right) under non-selective conditions.

In the course of clonal micropropagation of the selected shoots (conditionally salt-tolerant, with a normal morphotype) we obtained 6 regenerative lines of callus origin. Four of these lines belonged to ‘stable’ group: 3 downy birch clones (6psh/s, 3psh/1s, 3psh/2s) and 1 Karelian birch clone (18k/s). Two of these lines belonged to ‘sensitive’ group: 1 dalecarlica birch clone (R2/s) and 1 Karelian birch clone (Yu/s) (figure 8).
Following the study, we have developed a birch selection scheme to improve its in vitro salinity tolerance (figure 9). The scheme includes a gradual selection of the clones (lines), which are most tolerant to sodium chloride salinity, and uses two in vitro model biotest systems: first an in vitro collection of birch clones, then the callus cultures.

The criteria for selection for salinity resistance (NaCl) were as follows: at stage I – the preservation rate of cultures capable of resuming growth and development (at least 50%); at stage II – the frequency of callus formation (at least 40%), the viability of primary callus cultures (at least 40%), and the frequency of shoot regeneration in the morphogenic callus (at least 15%).

Complex testing of selected stable lines (biochemical, molecular-genetic, cytogenetic, etc.) should be carried out twice – at stage II (in vitro) and at stage III (ex vitro) of selection and testing of the material obtained.

![Figure 8. General view of regenerative birch lines selected in vitro in callus cultures under NaCl salinity stress conditions (0.5%).](image)

**Figure 8.** General view of regenerative birch lines selected in vitro in callus cultures under NaCl salinity stress conditions (0.5%).

The genetic variability (natural or induced) of the original plant is known to be the basis of successful breeding for stress resistance [4,6,18]. In addition, in vitro cultivation in itself (including the long-term one) can cause genetic changes (somaclonal variation) of cells and tissues [18].

![Figure 9. The in vitro selection scheme for birch tolerance to salinity stress.](image)

**Figure 9.** The in vitro selection scheme for birch tolerance to salinity stress.
In our research, we used the genetic variation of two in vitro model biotest systems: genetic diversity presented in our in vitro collection of birch clones (various species, varieties, hybrids, polyploids), as well as in the cellular heterogeneity of induced callus cultures.

In the experiment, we found that polyploid birch clones (2n=4x=56, 2n=3x=42) were more resistant to salination compared to diploid ones (2n=28). Previously, we had found that downy birch clones (6psh, 3psh, and 2psh) are tetraploid (2n=4x=56) (figure 10), which is typical of this species, whereas clones of silver birch (P1), Karelian birch (Yu), and dalecarlica birch (R2) are diploid (2n=2x=28), and 18k clone of Karelian birch is of diploid-triploid mixoploidy type (2n=42, 2n=28) [20,27].

The study showed that the two tetraploid clones of downy birch (3psh and 6psh) had the greatest salinity tolerance. It should be mentioned that the third tetraploid clone of downy birch (2psh) under similar salinity conditions showed poor salinity tolerance. Apparently, salt tolerance is determined not only by the ploidy level status, but also by the genotypic characteristics of the clone. The microsatellite analysis conducted earlier had shown that each of the studied birch clones had a unique genotype [28].

Our findings are consistent with literature data [29,30]. Thus, for instance, Cseri et al. [29] have shown that an increase in genome size can be an important source of genetic variability of Salix spp. Tetraploid willow plants (2n=76) on the soil mixed with crystal 1.5 g NaCl/kg soil exhibited better salt tolerance than the diploid ones (2n=38) (the scientists evaluated the plants’ biomass, leaf and root weights).

Moreover, our studies conducted on birch plants confirm that in vitro selection can be based not only on the original genetic variability of the plant (levels of ploidy, specifically), but also on the variability induced in the process of in vitro cultivation. Thus, we selected a stable salinity-resistant regenerative line of Karelian birch (18k/s) from a long-cultivated (for 26 years) triploid clone (18k) with a pronounced mixoploidy nature. The percent of cells with a modal triploid set of chromosomes (2n=42) was on average 61.8% (with variations for individual plants from 51.6 to 83.8%), diploid – 26.5%, and aneuploid – 11.7% [20]. Apparently, this pronounced genetic heterogeneity of the cell population of the clone allowed us to isolate the stable regenerative line using selective conditions. Therefore, mixoploidy (the presence of cells with different levels of ploidy in the tissue of one organism) can be considered as a source material for in vitro cell and tissue selection.

We used callus cultures obtained in vitro from pre-selected stable clones as further source of genetic variation increase.

Most researchers believe that in vitro callus formation is the result of induced reprogramming, de-differentiation of explant cells into stem (pluripotent) cells [23,24,31]. Callus, which initially is a mass of uniform cells, is gradually developing into systems of heterogeneous cells that differ in

---

**Figure 10.** Metaphase plates with the modal number of chromosomes of root meristem cells of birch microplants: diploid (2n=28) – P1 clone of silver birch (a) and R2 clone of dalecarlica birch (b); triploid (2n=42) – 18k clone of Karelian birch (c); tetraploid (2n=56) – 3psh clone of downy birch (d) [20,27].
morphology, colour, ploidy, and genetic characteristics. Moreover, each system of cells on an in vitro regeneration medium can develop in different ways [18,24]. Besides, depending on the ratio of endogenous and exogenous factors, in vitro cultivation can result in a broader range of morphogenetic scenarios than natural in planta conditions [4,24].

Using callus cultures and selective conditions (NaCl, 0.5%), we have successfully selected birch lines tolerant to in vitro salinity not only in the ‘stable’ group but also in the ‘sensitive’ group of clones (R2/s, Yu/s).

4. Conclusion
Our study has shown that in vitro selection offers great opportunities for increasing the stress tolerance of birch and creating a promising source material for adaptive selection. Biotesting, based on the assessment of the reactions of various birch clones and their calluses as a response to stress simulated in vitro, ensures differentiation and selection of the most resistant lines. We have identified two informative, relatively simple and reproducible indicators for the selection of birch lines most tolerant to NaCl salinity: the frequency of callus formation and the viability of callus cultures. These indicators characterized the initial and final stages of callus formation of primary explants. Their values were considerably higher in the group of ‘stable’ microclones. In both groups, the most viable and morphogenic calluses were green, dense and had globular structure.

Our study presents the in vitro selection scheme for birch tolerance to salinity stress. We also selected the most tolerant to NaCl salinity regenerative lines of birch through selective nutrient media. In the future, we plan to conduct a comprehensive evaluation and to test the selected lines in the field (ex vitro) to confirm the reproducibility and stability of the results obtained.

Acknowledgments
This research was supported by the Federal Forestry Agency of the Russian Federation (state assignment No. AAAA-A20-120012890092-6).

References
[1] Chen S, Hawighorst P, Sun J and Polle A 2014 Salt tolerance in Populus: Significance of stress signaling networks, mycorrhization, and soil amendments for cellular and whole-plant nutrition. *Environ. Exp. Bot.* 107 113 doi: 10.1016/j.envexpbot.2014.06.001
[2] Hasanuzzaman M and Tanveer M 2020 *Salt and Drought Stress Tolerance in plants: Signaling Networks and Adaptive Mechanisms* (Cham: Springer Nature Switzerland) p 413
[3] Yadav S and Sharma K D 2016 Molecular and Morphophysiological Analysis of Drought Stress in Plants *Plant growth* eds E. Rigobelo (London: IntechOpen Limited) chapter 10 pp 149-173
[4] Kruglova N N, Seldimirova O A and Zinatullina A E 2018 In vitro callus as a model system for the study of plant stress-resistance to abiotic factors (on the Example of Cereals). *Biology Bulletin Reviews* 138(3) 283 doi: 10.1134/S2079086418060063
[5] Munns R and Tester M 2008 Mechanisms of salinity tolerance. *Annu. Rev. Plant. Biol.* 59(1) 651 doi: 10.1146/annurev.arplant.59.032607.092911
[6] Rai M K, Kalia R K, Singh R, Gangola M P and Dhawan A K 2011 Developing stress tolerant plants through in vitro selection – An overview of the recent progress. *Environ. Exp. Bot.* 71(1) 89 doi: 10.1016/j.envexpbot.2010.10.021
[7] Terletskaya N, Khailenko N and Zhambakin K 2013 Stability of cereal crops to drought and saline stress in vivo and in vitro. *Journal of Life Sciences* 7(2) 135 [In Russian]
[8] Dasgupta M, Sahoo M R, Kole P C and Mukherjee A 2008 Evaluation of orange-fleshed sweet potato (*Ipomoea batatas* L.) genotypes for salt tolerance through shoot apex culture under in vitro NaCl mediated salinity stress conditions. *Plant. Cell. Tiss. Organ. Cult.* 94(2) 161 doi: org/10.1007/s11240-008-9400-2
[9] Khudolieieva L and Kutsokon N 2018 In vitro evaluation of salt tolerance of poplars and
willows. ScienceRise: Biological Science 2(11) 35 [In Ukrainen]

[10] Jan N, Qazi H A, Ramzan S and John R 2018 Developing Stress-Tolerant Plants Through in Vitro Tissue Culture: Family Brassicaceae Biotechnologies of Crop Improvement eds S. Gosal, S.Wani (Cham: Springer) chapter 1 pp 327-372

[11] Shupletsova O N and Shechenikova I N 2016 Results of using cell technologies for creation of new barley varieties resistant against aluminum toxicity and drought. Vavilov Journal of Genetics and Breeding 20(5) 623 doi: 10.18699/VJ16.183

[12] Dubrovna O V 2017 In vitro selection of wheat for resistance to abiotic stress factors. Plant Physiology and Genetics 49(4) 279 [In Russian]

[13] Yegorova N A and Stavtseva I V 2020 Optimization of the methods of lavender cell selection for resistance to low temperature stress. Proc. Int. conf. ‘Current state, problems and prospects of the development of agrarian science’ (Simferopol: ARIAL) p 175

[14] Fernández R, Bertrand A, Casares A, García R, González A. and Tames R S 2008 Cadmium accumulation and its effect on the in vitro growth of woody fleabane and mycorrhized white birch. Environ. Pollut. 152(3) 522 doi: 10.1016/j.envpol.2007.07.011

[15] Kuznetsova T Yu, Titov A F and Vetchinnikova L V 2008 Influence of cadmium on morphophysiological characteristics of birch in vitro. Lesnoy Zhurnal (Russian Forestry Journal) 3 40 [In Russian]

[16] Vuksanovic V, Kovacevic B, Kebert M, Katanic M, Pavlovic L, Kesić L and Orlovic S 2019 Clone specificity of white poplar (Populus alba L.) acidity tolerance in vitro. Fresen. Environ. Bull. 11(28) 8307

[17] Shao F, Zhang L, Wilson and Qiu D 2018 Transcriptomic analysis of Betula halophila in response to salt stress. Int. J. Mol. Sci. 19(11) 3412 doi: 10.3390/ijms19113412

[18] Kunahk V A 2011 Plasticity of the somatic cell genome and plant adaptability. Molecular and applied genetics 12 7

[19] Tabatskaya T M, Mashkina O S and Korchagin O M 2020 In vitro modelling of salinity stress for the selection of stress-tolerant birch lines. E3S Web. Conf. 224 04013 doi: 10.1051/e3sconf/202022404013

[20] Tabatskaya T M and Mashkina O S 2020 An experiment of a long-term preservation of a valuable birch genotypes collection using non-hormone nutrient media. Lesovedenie 2 147 [In Russian]

[21] Murashige T and Skoog F 1962 A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum 15(13) 473 doi: 10.1111/j.1399-3054.1962.tb08052.x

[22] Amineva E Yu, Tabatskaya TM, Mashkina O S and Popov V N 2017 Assessment of drought resistance of individual genotypes of Pinus sylvestris L. on the basis of in vitro tissue culture method in simulated stress conditions. Proceedings of the Saint Petersburg Forestry Research Institute 1 14 [In Russian]

[23] Ikeuchi M, Favero D S, Sakamoto Y, Iwase A, Coleman D, Rymen B and Sugimoto K 2019 Molecular mechanisms of plant regeneration. Annu. Rev. Plant. Biol. 70 377 doi: 10.1146/annurev-arplant-050718-100434

[24] Zinatullina A E 2020 Cytophysiological features of contrast callus types in vitro Uspekhi Sovremennoi Biologii 140(2) 183 [In Russian]

[25] Naaz A, Hussain S A, Naz R, Anis M, Alatar А А and Naaz F 2019 Successful plant regeneration system via de novo organogenesis in Syzygium cumini (L.) Skeels: an important medical tree. Agroforest. Syst. 93 1285 doi: 10.1007/s10457-018-0236-4

[26] Zair I, Chlyah A and Sabounji K 2003 Salt tolerance improvement in some wheat cultivars after application of in vitro selection pressure. Plant. Cell. Tiss. Org. Cult. 73(3) 237 doi: 10.1023/A:1023014328638

[27] Mashkina O S and Tabatskaya T M 2020 Morphogenesis of a dissected birch leaf in vitro culture. Russ. J. Dev. Biol. 51 397 doi: 10.1134/S1062360420060053

[28] Grodetskaya T A, Rzhevsky S G, Fedulova T P, Tabatskaya T M and Mashkina O S 2018
Identification of *Betula pendula* Roth var. *carelica* and *Betula pubescens* Ehrh. genotypes with the use of microsatellite markers. *Proceedings of Voronezh State University. Series: Chemistry. Biology. Pharmacy* 3 121 [In Russian]

[29] Cseri A, Borbely P, Poor P, Feher A, Sass L, Jancso M, Penczi A, Radi F, Gyuricza C, Digruber T and Dudits D 2020 Increased adaptation of an energy willow cultivar to soil salinity by duplication of its genome size. *Biomass. Bioenerg.* 140 105655 doi: 10.1016/j.biombioe.2020.105655

[30] Xue H, Zhang F, Zhang Z-H, Fu J-F, Wang F, Zhang B and Ma Y 2015 Differences in salt tolerance between diploid and autotetraploid apple seedlings exposed to salt stress. *Sci. Hortic-Amsterdam.* 190 24 doi: 10.1016/j.scienta.2015.04.009

[31] Feher A 2019 Callus, dedifferentiation, totipotency, somatic embryogenesis: what these terms mean in the era of molecular plant biology? *Front. Plant. Sci.* 10 536 doi: 10.3389/fpls.2019.00536