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Silver birch (*Betula pendula* Roth.) culture initiation in vitro and genotype determined differences in micropropagation

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Abstract
Micropropagation has several advantages over conventional vegetative propagation methods, but it is limited by genotype responsiveness. We assessed the effect of age of the mother-tree and the time of explant collection on culture initiation, as well as the multiplication ability and effect of different nutrient media and plant growth regulators on silver birch genotypes. Explants collected from 1-year-old trees (66%) and explants collected in spring (64–67%) developed a significantly (both $p < 0.001$) higher proportion of shoots than those from 15-year-old trees (39%) and those collected in mid-summer (31%) and autumn (29%), respectively. In a stabilised culture, the length of the main shoot varied from 1.3 to 7.8 cm between genotypes, and the multiplication rate ranged from 1.0 to 6.8 shoots per explant. Hyperhydrated shoots were present in 17 out of 50 clones, and, among the clones, ranged from 14 to 50%. Cultures on the Murashige and Skoog basal medium had a higher multiplication rate than cultures on a Woody Plant Medium, and the application of zeatin provided better results than 6-benzylaminopurine. The difference between cytokinin types was 11–29% for the multiplication rate and 21–29% for the length of the main stem. The highest multiplication rate was obtained using a zeatin concentration of 0.5 mg L^{-1}. However, better shoot growth and proliferation had a significant positive relation to shoot hyperhydration (all $p < 0.001$). Therefore, a medium with an optimal balance between the multiplication rate and the number of hyperhydrated shoots should be carefully selected.

Keywords *Betula pendula* · Genotype · Micropropagation · Culture initiation · Cytokinin

Introduction

Wood is the most versatile renewable material that is used to substitute for fossil resources from construction wood and packaging boards to textiles and biochemical production. The growth of population and middle-class income, as well as bioeconomic development have steadily increased the global demand for wood and wood-based products: wood demand is forecasted to increase more than twice from 2010 to 2030, and more than thrice from 2010

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to 2050 (WWF 2012), while roundwood production under the continuous trend is forecasted to increase by 16% from 2018 to 2050 (Hetemäki et al. 2020). The increase in the productivity of native species and the establishment of fast-growing species and genotypes plantations are strategies to ensure a sufficient wood supply (Mola-Yudego et al. 2017). In both respects, silver birch (*Betula pendula* Roth) has potential in the Northern European and Baltic states (Rytter et al. 2013). Here, birch is one of the most abundant tree species (Brus et al. 2012) and is highly productive both in forest sites and on abandoned agricultural land (Uri et al. 2012; Lutter et al. 2015). It has several advantages over other fast-growing species that are suitable for the region, especially concerning environmental risks, due to its high tolerance and adaptation capacity to a large variety of climates and soils (Dubois et al. 2020). In contrast, spruce (*Picea abies* (L.) Karst.) is prone to wind, drought, and bark beetles (Mezei et al. 2017). Moreover, these disturbances are increasing in frequency (Seidl et al. 2017). *Populus* species and their hybrids are preferred by herbivores. In addition, due to their non-native origin (except for *P. tremula*), these species might be damaged by abiotic factors, such as early autumn frost (Lazdiņa et al. 2016; Šēnhofa et al. 2016). The *Salix* species or genotypes may suffer from frost- and bacteria-induced dieback (Cambours et al. 2005). Moreover, this species has limited wood-processing possibilities due to stem size.

Size and external quality traits, including the absence of defects (cracks) in the stem, have a substantial effect on the financial return for birch (Kilpeläinen et al. 2011; Viherä-Aarnio and Velling 2017). High-quality timber is a valuable resource for plywood, sawnwood, and wood-based panel production, whereas lower-grade timber is used as pulpwood, and the residue is utilised for bioenergy. Planting is the preferred stand establishment method for the intended management of a high-quality plantation (Hynynen et al. 2010). However, birch is primarily established via natural regeneration. For instance, in Latvia, only 11% of the area regenerated with birch is planted (Valsts meža dienests 2019). Although planting requires a higher initial investment, artificial seedlings have more vigorous growth in comparison to naturally regenerated seedlings; thus, they are more competitive with ground vegetation and require less intensive management, i.e. less frequent tending, in young stands (Hynynen et al. 2010). Moreover, planting allows the use of genotypes with enhanced desired characteristics because the seedlings originate from improved material, i.e. seeds collected from seed orchards (Gailis et al. 2012).

Silver birch has considerable ecological adaptability and climatic differentiation, which are related to its wide distribution (Koski and Rousi 2005; Rousi et al. 2011), thus providing a good basis for breeding. To improve the stem quality, the phenotypic selection of plus-trees is based on stem straightness, apical dominance, and desirable branch properties, including good natural pruning (Koski and Rousi 2005; Stener and Jansson 2005), yet breeding aims to balance between increased growth, timber quality, and resistance to biotic and abiotic factors. Several studies have affirmed the economic gain from the use of improved regeneration material (Jansson et al. 2017). In Latvia, the breeding of birch trees started in 1995 and has substantially improved the yield and stem quality (Jansons et al. 2011; Zeltiņš et al. 2018). The selection of the top 10% of families in the progeny trials at the age of 14 years resulted in a genetic gain of 10–27% for height and diameter at breast height, accounting for 26–62% of the stem volume. The gain in the overall stem quality was 6–10%, with improved straightness by 9–21% and branch diameter and angle by 2–18% (Gailis et al. 2020a). Furthermore, the occurrence of stem defects, such as spike knots, double tops, and lost tops, were more affected by environmental factors than genetic control, and low-to-moderate genetic correlations between growth and stem quality traits indicated the potential to improve productivity and wood quality simultaneously. As

a result, the use of improved planting material significantly increased the economic gain from commercial thinning (Gailis et al. 2020b).

Breeding is performed in repeated cycles of selection of the best-performing genotypes and their recombination using controlled pollination and progeny testing (Ruotsalainen 2014). The establishment of clonal collections and seed orchards can be accelerated when vegetative propagation is used. Moreover, vegetative propagation allows the maintenance of all inherent traits of the mother-tree (Ryynänen and Aronen 2005) and produces uniform-sized plantlets (Jones et al. 1996). Such trees reach the target diameter for final felling sooner (Zeltiņš et al. 2018), and any time-related damage is lower (Donis et al. 2020): as the vegetative propagation ensures a way to propagate the best-growing genotypes, time (and, consequently, the probability of damages) to reach similar dimensions of conventionally propagated trees can be reduced. However, using conventional vegetative propagation methods on mature birch has not been successful. Cuttings can be rooted only up to the age of five years, and grafting is limited by the incompatibility of rootstock and scion and delayed graft failure (Ryynänen and Ryynänen 1986; Welander 1993; Mikola 2009). Therefore, micropropagation can be used as an alternative method with several advantages: numerous pathogen-free plantlets can be obtained in a short period and propagation can be done throughout the year regardless of the season (Jones et al. 1996; McCown 2000; Ryynänen and Aronen 2005).

Yet, micropropagation per se does not infer a general advantage over seed-born plants, and careful selection of clones to be multiplied has crucial importance on breeding gain (Viherä-Aarnio and Velling 2001). Micropropagation is a laborious process that involves skilful manual work in an aseptic laboratory and therefore results in higher costs than conventional nursery practice (Koski and Rousi 2005). The success of culture initiation depends on the explant type and its position on the stock plant, the age of the stock plant, the tree species, and variations among trees of the same species (Welander 1993). Not all genotypes multiply with equal success (Koski and Rousi, 2005), but multiplication is largely dependent on the applied nutrient media and plant growth regulators (Jokinen and Törmälä 1991), which must be determined experimentally.

This study aimed to assess the effect of the mother-tree age and the time of the explant collection on the culture initiation in vitro. This is followed by an assessment of the reproduction capacity of different genotypes and the optimal medium content.

Materials and methods

The study material was collected from a 15-year-old progeny trial in Rembate (56°44′ N, 24°49′ E) that was established using open-pollinated (half-sib) families of silver birch (*Betula pendula* Roth) plus-trees phenotypically selected across forests in Latvia (55°40′–58°05′ N, 20°58′–28°14′ E). The trial was established on former agricultural land with mesotrophic, mesic, and silty soil, corresponding to the forest site type *Oxalidosa* (according to the classification by Bušs (1997)). One-year-old containerised seedlings were planted in a randomised complete block design with four-row plots of 32 trees per row for each family in three to five replications at initial density of 2500 trees ha^{-1} (2 × 2 m).

To assess the effect of the mother-tree age on shoot initiation in vitro, the explants from clone 54–95 in Rembate were compared with the explants from one-year-old grafted scions (one year after grafting on two-year-old rootstock) of the same clone in Kalsnava (56°40′ N, 25°58′ E), and all samples were collected in March. To assess the effect of the time of

the explant collection, twigs of clone 54-95 from Rembate were collected from the lowest part of the canopy four times per year: in March and April (spring), and in June (mid-summer) and September (autumn). In total, 100 stem segments per each ontogenetic age and collection time were initiated in vitro.

In laboratory, stem segments containing one bud were excised and gently washed with a toothbrush and dish soap under running tap water, and rinsed toughly. Stem segments were sterilised for 10 min in 0.1% $HgCl_2$ with a few drops of Tween 20, and rinsed three times with distilled water. The stem segments were inserted into glass test tubes (18 × 180 mm, with a metal cap) containing 3 mL of woody plant medium (WPM; Lloyd and McCown 1980) supplemented with WPM micronutrients, WPM vitamins, 1.0 mg L^{-1} of 6-benzylaminopurine (BAP), 0.05 mg L^{-1} of naphthaleneacetic acid, 20 g L^{-1} of sucrose, and 6 g L^{-1} of agar. The pH of the medium was adjusted to 5.8, and the tubes were autoclaved for 15 min (110 kPa, 121 °C). The culture was incubated at 25 ± 3 °C under a 16-h photoperiod of cool-white fluorescent light (photosynthetically active radiation with photon flux density at 140–160 µmol m^{-2} s^{-1}). After 30 days, the proportion of initiated shoots was evaluated.

The multiplication rate and the growth of shoots between 50 genotypes in Rembate were assessed using a stabilised in vitro culture. Ten shoots per each clone were inserted into test tubes (one explant per tube) containing 3 mL of Murashige and Skoog (MS) basal media (Murashige and Skoog 1962), supplemented with MS micronutrients, 0.2 mg L^{-1} of zeatin, MS vitamins, 20 g L^{-1} of sucrose, and 6 g L^{-1} of agar. The pH of the medium and the cultivation conditions were set as described for the culture initiation. After 30 days, 500 shoots were assessed. The main shoot was measured. The lateral shoots ≥ 0.5 cm were counted, and the multiplication rate of each shoot was determined by the number of 1.5-cm-long shoot fragments (hyperhydrated shoots were not counted) that could be obtained from one plant. The total multiplication rate and occurrence of hyperhydrated shoots were noted. According to the growth parameters, the clones were divided into four groups to assess the effect of the macronutrient media and plant growth regulators. Group divisions were based on the multiplication rate and ability to proliferate. Group 1 has a multiplication rate of 3.4–6.8. Group 2 has a multiplication rate of 1.7–3.3. Group 3 has a multiplication rate of 0–1.6 with proliferation, and Group 4 has a multiplication rate of 0–1.6 without proliferation.

Three clones from each group (24 shoots per clone in each treatment) were randomly selected to test the effect of the multiplication medium and cytokinin type and concentration in the following combinations: (1) WPM, zeatin 0.1 mg L^{-1}, (2) WPM, zeatin 0.5 mg L^{-1}, (3) MS, zeatin 0.1 mg L^{-1}, (4) MS, zeatin 0.5 mg L^{-1}, (5) MS, zeatin 1.0 mg L^{-1}, (6) MS, BAP 0.5 mg L^{-1}, and (7) MS, BAP 1.0 mg L^{-1}. The pH of the medium and the cultivation conditions were set as described for the culture initiation. After 30 days, 2016 shoots were assessed. The main and lateral shoots were measured. The lateral shoots ≥ 0.5 cm were counted. The multiplication rate of each shoot was determined by the number of 1.5-cm-long shoot fragments (hyperhydrated shoots were not counted) that could be obtained from one plant. Finally, the total multiplication rate and occurrence of the hyperhydrated shoots were noted.

We used the chi-square (χ^2) test to assess the distribution of the initiated shoots between the time of explant collection and the age of the mother-tree. The analysis of variance and Tukey's honestly significant difference test were used to assess the differences in the length of the main shoots, the length and number of lateral shoots, the multiplication rate, and proportion of the hyperhydrated shoots between the clones and groups of clones. We used generalised linear models with a Poisson distribution to assess the relationship between

the length of the main shoot and the number of the lateral shoots, as well as to evaluate the effect that the length of the main shoot, number of lateral shoots, length of lateral shoots, and total multiplication rate had on the number of hyperhydrated shoots. Linear model was used to assess relationship between the length of the main shoot and the length of the lateral shoots. The regression coefficients ± standard error are shown. All calculations were done in R 3.5.1. (R core team 2018). In addition, all tests were performed at $\alpha = 0.05$.

Results

The age of the mother-tree and explant collection time had a significant effect (both $p < 0.001$) on the shoot initiation in vitro (Fig. 1). The explant collected from the one-year-old trees had 66% initiated shoots, and the explant collected from the 15-year-old trees had 39% of initiated shoots. The explants collected in spring developed a higher proportion of initiated shoots (64% in March and 67% in April) than explants collected in June and September (31% and 29% of shoots initiated, respectively).

In the stabilised culture, the growth parameters greatly differed between the clones. The multiplication rate varied from 1.0 (no multiplication) to 6.8 shoots per explant (Fig. 2), and the lengths of the main shoots varied from 1.3 to 7.8 cm (Fig. 3). Moreover, the number of lateral shoots varied from 0 to 3.8 per explant.

Clones exhibited different growth patterns. Several genotypes had a large main shoot but a low number of lateral shoots (e.g. clone L 29, Fig. 3a), whereas others had a short main stem but a high number of lateral shoots (e.g. clone Bau 40-13, Fig. 3a). A high multiplication rate was achieved by both of these patterns and by the intermedium between them. Regardless of the large variation, the number of lateral shoots showed a negative relationship to the length of the main stem ($p < 0.01$, regression coefficient -0.11 ± 0.04). Almost a quarter of the clones had a low multiplication rate; 12 clones had a mean multiplication rate lower than two, and seven clones did not multiply.

A high proportion (50%) of hyperhydrated shoots was present for two clones, one among the clones with a high multiplication rate (clone 54-616-783, Fig. 2a) and the other

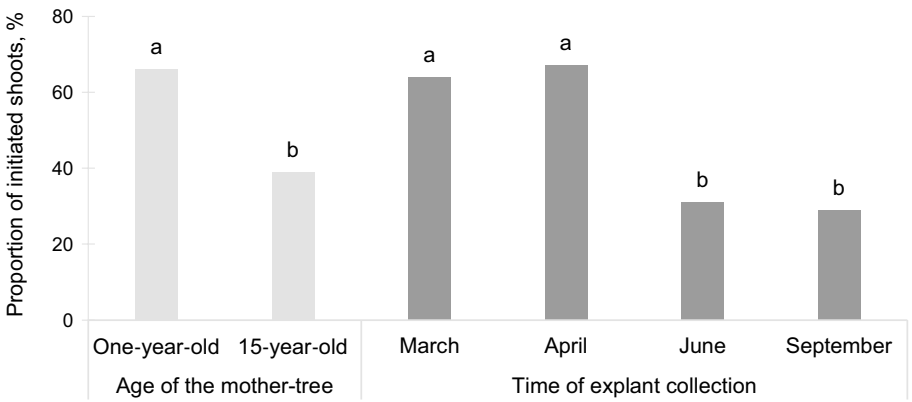

Fig. 1 The proportion of initiated shoots according to the age of the mother-tree and explant collection time. Statistically significant differences between groups of age and between groups of collection time are denoted with different letters (both $p \leq 0.001$)

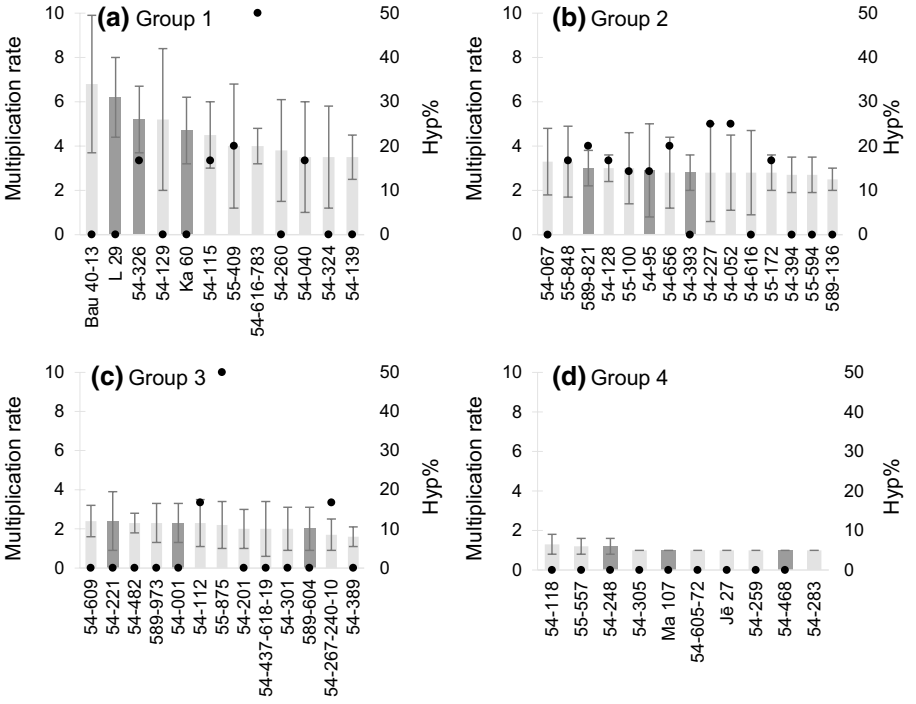

Fig. 2 The multiplication rate (bars; ± standard error) and proportion of hyperhydrated shoots (Hyp%; bullets) among the clones in (**a**) Group 1, (**b**) Group 2, (**c**) Group 3, and (**d**) Group 4. Clones that were selected to test the effect of different treatments are dark grey

with a low multiplication rate (clone 55-875, Fig. 3). A moderate proportion (14–25%) of the hyperhydrated shoots was present for 15 out of 50 clones. The number of hyperhydrated shoots demonstrated no relationship (both $p > 0.05$) to the length of the main stem and number of lateral shoots. However, a positive relationship ($p < 0.001$, regression coefficient 0.56 ± 0.04) exists between the number of hyperhydrated shoots and the total multiplication rate.

The mean multiplication rate between the treatments varied from 1.8 to 6.7 (mean 3.9). Within all groups, the culture on the MS medium exhibited a higher multiplication rate (Fig. 4) and a longer length of the main stem than the culture on the WPM. For these two parameters, the difference between the media type was most pronounced for Group 1 (Fig. 4a) and gradually decreased for Group 4 (Fig. 4d). However, the culture on the WPM had a substantially lower proportion of hyperhydrated shoots than the culture on the MS medium, and it ranged from 0 to 6.3 (mean 2.9) for WPM and from 0 to 38.0 (mean 13.2) for the MS medium. The number and length of the lateral shoots had no clear relation to the media type.

The length of the main shoot showed a significant relation to the length of the lateral shoots ($p < 0.001$, regression coefficient 1.68 ± 0.24) and the number of lateral shoots ($p < 0.01$, regression coefficient 0.23 ± 0.06). The length of the main shoot had a consistent ranking between the groups of clones (Fig. 5a). However, the number of lateral shoots highly fluctuated between treatments (Fig. 5b), affecting the ranking of the multiplication rate (Fig. 5c). Regardless of the media and cytokinin type, both the

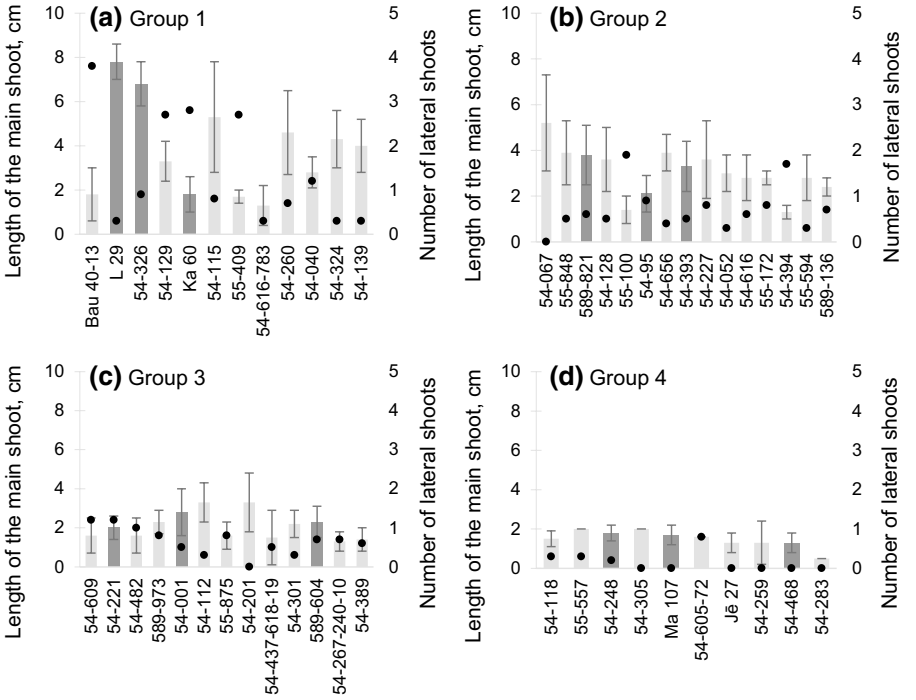

Fig. 3 Length of the main shoot (bars; ± standard error) and the number of lateral shoots (bullets) among the clones in (**a**) Group 1, (**b**) Group 2, (**c**) Group 3, and (**d**) Group 4. Clones that were selected to test the effect of different treatments are dark grey

multiplication rate and length of the main stem were the highest in Group 3, followed by Group 2 and then Group 1. As expected, Group 4 contained clones with the poorest growth among the genotypes and had the poorest results for all growth parameters (Figs. 4 and 5). The number of hyperhydrated shoots showed a positive relation with all shoot growth parameters: the length of the main stem ($p < 0.001$, regression coefficient 0.77 ± 0.07), the number ($p < 0.001$, regression coefficient 3.11 ± 0.07), and length of the lateral shoots ($p < 0.001$, regression coefficient 0.98 ± 0.21). Consequently, a significant relation ($p < 0.001$, regression coefficient 0.44 ± 0.02) between the number of hyperhydrated shoots and the total multiplication rate exists.

The effect of the cytokinin type and concentration on the growth parameters was assessed separately on the WPM and MS medium. On the MS medium, regardless of the concentration, zeatin exhibited better results on the multiplication rate and length of the main stem than BAP for all groups (Fig. 4). Within the groups, the mean difference between the zeatin and BAP treatments was 11–29% for the multiplication rate and 21–29% for the length of the main stem. Moreover, within all groups, the treatments with zeatin had a lower mean proportion of hyperhydrated shoots (ranging from 2.4 to 23.4%) than the treatment with BAP (ranging from 11.1 to 31.8%). Among the MS and zeatin treatments, the highest multiplication rate was obtained with a concentration of 0.5 mg L^{-1} in total, and for all groups except Group 1 (Fig. 4). This concentration also resulted in the highest mean length of the main stem within all groups.

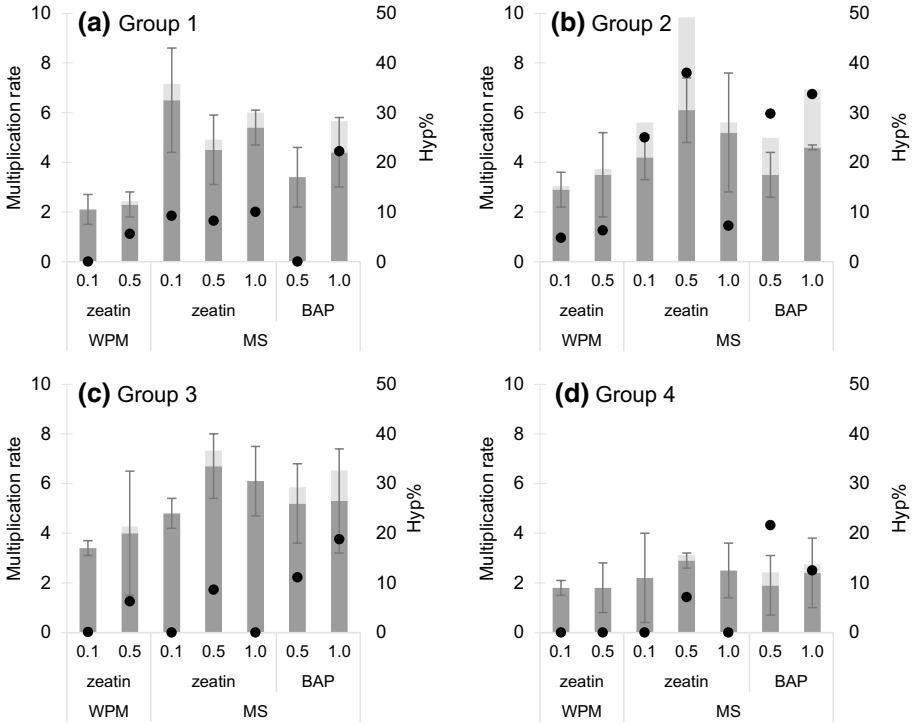

Fig. 4 Stacked multiplication rate (dark grey; ± standard error) and number of hyperhydrated shoots (light grey), and proportion of hyperhydrated shoots (Hyp%, bullets) according to the media content in (**a**) Group 1, (**b**) Group 2, (**c**) Group 3, and (**d**) Group 4. WPM, woody plat medium; MS, Murashige and Skoog basal media; BAP, 6-benzylaminopurine

On the WPM, the zeatin at a concentration of 0.5 mg L^{-1} had an equal or slightly higher multiplication rate and a longer length of shoots than zeatin at a concentration of 0.1 mg L^{-1}, but these differences were more negligible than on the MS medium. The number of lateral shoots between the different treatments varied from 0.1 to 1.2 (mean 0.6). No specific relation between the length and number of lateral shoots and cytokinin type was observed.

Discussion

Culture initiation in vitro is the most important stage in micropropagation because the outcome determines further operation possibilities. However, it is also the most problematic stage in the micropropagation of perennial plants. Recalcitrance (i.e. the inability of plant cells, tissues, and organs to respond to in vitro manipulations) could be a major limiting factor in the application of in vitro propagation (Benson 2000). Several factors, such as the collection time, age of the stock plant, bud position in the mother-tree crown, genotype, pre-treatment storage time, conditions, and medium content (Welander 1993, 1988; McCown 2000; Vaičiukynė et al. 2017), affect the responsiveness of the plant material.

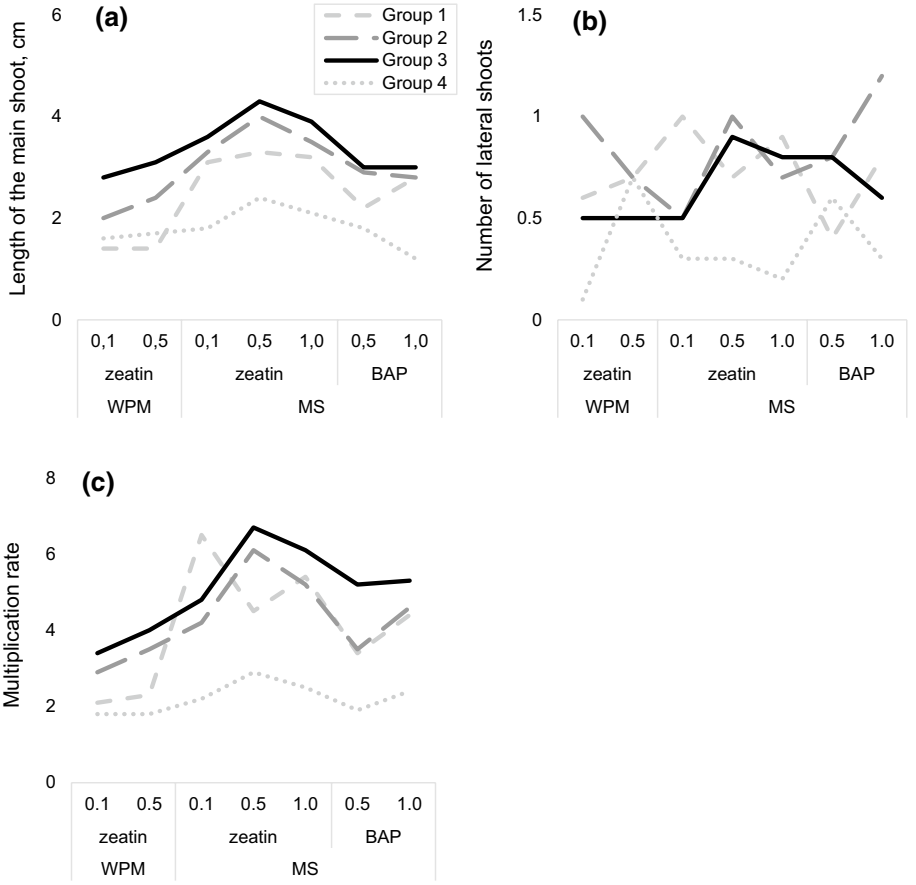

Fig. 5 Group mean (**a**) length of the main shoot, (**b**) number of lateral shoots, and (**c**) multiplication rate according to the media content. WPM, woody plat medium; MS, Murashige and Skoog basal media; BAP, 6-benzylaminopurine

Plants have complex life cycles, associated with reproduction, vegetative development, and morphogenesis. Temperate species undergo cycles of dormancy that determine periods of active shoot growth and cell division. Consequently, plants exhibit seasonal differences in their responses to tissue culture, depending on the time of the year when the explants are procured (Benson 2000). We obtained the highest proportion (about 65%) of initiated shoots from the explants collected in spring (Fig. 1). About one-third of the explants collected in mid-summer and autumn developed shoots, although the initiation rate was significantly lower than that in spring. This result corresponds well with the general pattern that cultures are most easily initiated from the explants collected in spring to early summer after a break of dormancy (George et al. 2008a).

A similar trend had been observed for other temperate tree species. *Populus tremula* L. had the highest survival rates of buds collected in late February and early March, while buds collected earlier had less intensive callus formation and plantlet development, and buds collected later had intense decay and infections (Peternel et al. 2009). For *Quercus robur* L., the most responsive explants were collected later, from May to

July (Civínová and Sladský 1990), which is probably related to their later bud burst in comparison to the aspen and birch (Linkosalo 2000; Lange et al. 2016). In contrast, others have found that the initiation of the silver birch culture was more successful from explants collected in autumn and winter (Jokinen and Törmälä 1991), and bud treatment that induces dormancy resulted in a higher proportion of buds developed into shoots (Welander 1993), suggesting that factors other than the growing season might be more significant for culture initiation.

Plant growth regulators applied during the initiation affect the induction of the shoot formation (Magnusson et al. 2009). Seasonal cycles of perennial plants determine the dynamics of the endogenous levels of growth regulators, which are responsible for the induction of dormancy or inhibit growth and further development. For instance, a low concentration of applied BAP (0.2 mg L^{-1}) resulted in a higher shoot number in *Q. robur* explants collected in February and March but with an increased BAP concentration (1 and 2 mg L^{-1}) better results were obtained from buds collected from May to July (Civínová and Sladský 1990). Moreover, buds collected from May to July had a higher regeneration capacity than those from February to April, but higher BAP concentrations were needed to stimulate their meristematic activity (Civínová and Sladský 1990).

Higher initiation success in spring might be related to the seasonally determined occurrence of microbes within the buds. For mature *B. lenta* L., dormant buds demonstrated lower contamination than buds collected in spring (Rathwell et al. 2016). Similar results were obtained for *Platanus occidentalis* L., and the contamination rate gradually increased from 14% in January to 48% in July (Tao et al. 2007). Spring was the most appropriate time for bud collection to reduce contamination in *Ulmus americana* L., whereas almost all were contaminated among dormant buds (Shukla et al. 2012). In contrast, low contamination from May to October and significantly higher contamination from December to April were found for *Pinus sylvestris* L., which is assumed to be related to better resistance of the tissue against pathogens during the active period (Hohtola 1988).

Trees exhibit developmental changes as plants progress from the juvenile to adult phases leading to a decline in their potential for micropropagation (von Aderkas and Bonga 2000). Juvenile seedling tissue is generally more responsive to culture initiation in vitro than that of mature trees. Juvenile seedlings are more easily initiated and grow and proliferate at a more rapid rate than adult material (George et al. 2008a). Cultures from mature trees are more problematic due to higher contamination rates, the browning of tissues, and recalcitrance (George et al. 2008a). One of the methods to obtain juvenile material is grafting, which can lead to a partial rejuvenation of the donor plant and can overcome recalcitrance in mature trees (Benson 2000). No assessment of true rejuvenation or reinvigoration of the scion (Wendling et al. 2014) was done in our study, yet, explants from the grafted one-year-old tree had a significantly higher proportion of initiated shoots than the explants from the 15-year-old tree (Fig. 1). Similarly, a high bud initiation (80%) was achieved using 3-year-old plants of *B. lenta*, whereas the explants from mature trees did not develop into shoots (Rathwell et al. 2016). However, most *Betula* species can be propagated from adult material without major difficulties (Welander 1993), and several studies found success using buds from mature birch trees (Ryynänen and Ryynänen 1986; Jones et al. 1996; Aubakirova and Kalashnikova 2011).

Our results are limited by using only one genotype (clone 54–95) to assess the effect of the explant collection time and age of the mother-tree. The initiation success is more affected by the genotype than the aforementioned factors (Jokinen and Törmälä 1991), but the genotype response is affected by the physiological heterogeneity of the ramets and buds of the same ramet (Civínová and Sladský 1990). However, these limiting factors could be

overcome if the genotype is generally competent for growing in vitro (Jokinen and Törmälä 1991).

Once the culture is fully stabilised, biologically responsive tissues show little seasonality and progression through phase states (McCown 2000). We observed large variations in the multiplication rate between and within genotypes (Fig. 3), ranging from 1.0 to 6.8 among the clones. Close results (a multiplication rate between 1.6 and 7.4) were found among 10 genotypes selected in Sweden, Finland, and Germany (Ewald et al. 2002). Large variations but higher multiplication rates (from 2 to 20) were observed between 100 genotypes by Jokinen and Törmälä (1991). Among our tested genotypes, 12 clones had a mean multiplication rate lower than two, and seven clones did not multiply. This might be related to the limited time of the study because four to ten weeks are required for bud induction in birch, depending on the explant type, age, and physiological conditions of the mother-tree and genotype (Welander 1993). Moreover, the applied medium and plant growth regulators affect genotype performance.

The optimal media composition is species- and genotype-specific, and while a number of plants exhibit decent growth in the range of media, others may have considerable differences in their performance (McCown and Sellmer 1987). Therefore, the number of genotypes that are possible to micropropagate could be increased by modifying the composition of the medium and applying different types and concentrations of cytokinins (Jokinen and Törmälä 1991). For optimal shoot growth in vitro, the optimal amount of minerals should be provided. The most commonly used basal media in micropropagation of birch are the MS and WPM basal media, although others are also used (Ewald et al. 2000; Iliev et al. 2003). We observed a slightly higher multiplication rate (Fig. 4) and longer length of the main stem for cultures on the MS medium than those on the WPM. Similarly, poorer growth on the WPM was shown for *B. lenta*. The shoots on WPM cultures had red pigmentation, were significantly shorter, and had fewer nodes compared to the MS cultures, although the multiplication rate was not significantly lower (Rathwell et al. 2016). They associated this response to ionic strength (i.e. salt concentration) in the basal salt mixture, as the amount of ammonium nitrate is about a quarter lower in the WPM than in the MS medium. A limiting effect of low nitrogen was revealed for a given genotype of *Populus* hybrid. At first, the cultures on the WPM had poor growth, whereas these cultures on the MS medium were maintainable indefinitely. However, after an increase in NH_4NO_2 on the WPM to the level of MS, growth improved substantially, and was similar to that observed for the MS medium (McCown and Sellmer 1987). In contrast, four genotypes of *Betula platyphylla* Sukatchev var. *japonica* (Miq.) Hara × *B. pendula* had a constantly higher multiplication rate on the WPM than on the MS medium, with the mean long-term multiplication rate on these media at 3.7 and 2.8, respectively (Meier-Dinkel 1992).

Plant growth regulators have the most important role in birch in vitro shoot initiation and cultivation. Among them, cytokinins are highly effective in stimulating cell division and the control of morphogenesis (George et al. 2008b). For shoot multiplication, the most commonly used cytokinins are single BAP or zeatin at concentrations of 0.2–5.0 mg L^{-1} and 1.0–5.0 mg L^{-1}, respectively, or BAP concentrations of 0.7–2.0 mg L^{-1} in combination with a low concentration of auxin (Meier-Dinkel 1992).

The effect of the particular cytokinin should be assessed experimentally, and the response is determined by the particular compound used, type of culture, genotype, and ontogenetic stage of the tissue (George et al. 2008b). For instance, two *B. pendula* genotypes demonstrated a distinct response to supplementation with cytokinin. One of them had a significant decrease in the shoot length and an increase in the number of shoots per explant, whereas the other did not affect these parameters. Simultaneously, the latter

genotype had a higher number of shoots per explant in both the control medium and the medium supplemented with BAP (Vaičiukynė et al. 2017). For several *Betula* species, the absence of cytokinins resulted in the poor growth of new shoots (Cheng et al. 2000) or no shoot development and explant death (Magnusson et al. 2009; Rathwell et al. 2016; Girgžde and Samsone 2017). Yet, typically, a narrow range of concentration achieves the best results. We observed slightly better results for the multiplication rate and length of the main stem on the media supplemented with zeatin instead of BAP. On the MS medium supplemented with zeatin, a concentration of 0.5 mg L^{-1} resulted in the highest mean length of the main stem within all groups and in the highest multiplication rate for three out of four groups (Fig. 4). On treatments supplemented with BAP, a higher concentration of cytokinin resulted in a somewhat higher multiplication rate than a lower concentration. The observed tendencies remained non-significant due to the large variation, although these tendencies were nearly constant between the groups of clones.

Several studies have tested optimal concentrations of cytokinins on *Betula* genotypes. The effect of the BAP concentration was tested on a cultivar of *B. platyphylla.* The concentration at 2.2 µM of BAP (rounded 0.5 mg L^{-1}) resulted in about a four-times higher number of new shoots than a concentration at 1.1 µM, and in a twice higher number of new shoots than the concentration at 4.4 µM (Cheng et al. 2000). For the *B. pendula* genotype, the main shoots on 1.0 mg L^{-1} of BAP had a similar length to those on treatments containing zeatin (0.5–1.0 mg L^{-1}). Yet, 1.0 mg L^{-1} of BAP had more than a two-fold higher number of lateral shoots and hence achieved a significantly higher multiplication rate than the other treatments (Girgžde and Samsone 2017). In addition, a study of *B. lenta* revealed a significantly higher result of shoot growth with BAP treatment compared to the supplementation of 2-isopentenyladenine (2-IP) and thidiazuron (TDZ), and the highest multiplication rate was achieved at a BAP concentration of 5.0 µM (rounded to 1.1 mg L^{-1}; Rathwell et al. 2016). For *B. platyphylla* and *B. papyrifera* Marsh, the BAP at a concentration of 10–20 µM (rounded to 2.3–4.5 mg L^{-1}) and TDZ at a concentration of 4–8 µM proliferated more shoots than other treatments (Magnusson et al. 2009). Different intensities of response to cytokinins might be related to the dynamics of the endogenous levels of growth regulators over the seasons, and thus could be related to the explant collection time. Civínová and Sladský (1990) found that *Q. robur* explants collected from May to July needed a higher concentration (2 mg L^{-1}) of BAP than those collected from February to April (0.5 mg L^{-1}) to achieve the highest regeneration capacity.

Within the tested media composition, groups of clones had a generally consistent ranking for the length of the main shoots (Fig. 5), but the number of lateral shoots fluctuated with the treatments, hence affecting the ranking of the multiplication rate. Moreover, groups of clones had reversed ranking in the multiplication rate in comparison to the first subculture (assessment of different genotypes, Figs. 2 and 3), although, typically, the multiplication rate remains stable during several subcultures (Jokinen and Törmälä 1991). The difference in the performance of the groups between subcultures indicates the importance of the careful selection of the media content.

A higher cytokinin concentration is beneficial until a certain study-specific threshold is reached, and afterwards, shoot abnormalities appear. The explant might form many small shoots that fail to elongate, and the leaves might have an unusual shape (George et al. 2008b). For instance, a high concentration of zeatin induced the spontaneous appearance of abnormal shoots in *B. pendula*. A concentration of 5 mg L^{-1} resulted in 3.4% fasciated shoots, and 10 mg L^{-1} resulted in 4.2% fasciated shoots, whereas all shoots appeared anatomically normal in the absence of or at a low concentration (2 mg L^{-1}; Iliev et al. 2003). In another study, at the highest tested BAP concentration (5.3 µM; rounded to 1.2 mg L^{-1}),

the shoots had variable sizes and appeared hyperhydrated and chlorotic (Cheng et al. 2000). Hyperhydricity (previously called 'vitrification') characterises frequently observed malformations during vegetative propagation in vitro, where the plants appear turgid and watery at their surface and are hypolignified (Gaspar 1991; Debergh et al. 1992). Their shoots have broad and thick stems; short internodes; and thick, frequently very elongated, wrinkled, curled, and brittle leaves (Franck et al. 1995).

In our study, 34% of the clones had hyperhydrated shoots (Fig. 3), ranging from none to 50% of the shoots. Hyperhydricity is related to the particular conditions of the in vitro culture and results from the inability of the shoots to adapt normally to the reactions caused by stress factors, e.g. wounding, high ionic strength, and inappropriate lighting and temperature (Kevers et al. 2004). In our study, BAP promoted more hyperhydration. A high proportion of hyperhydrated shoots was also present for zeatin at a concentration of 0.5 mg L^{-1}, i.e. for treatment that resulted in the highest multiplication rate. Overall, the number of hyperhydrated shoots was positively related to better shoot growth and proliferation. These results agree with the study conducted by Gaspar (1991), who reported that hyperhydricity is related to intensive multiplication, i.e. frequent subcultures with a high rate of regeneration, but is not affected by the physiological conditions of the mother-tree at the time of the explant collection.

Conclusions

We aimed to assess the effect of the mother-tree age and explant collection time on the culture initiation in vitro, as well as to assess the multiplication capacity of different genotypes and the optimal media content on stabilised cultures. For initiation, explants from ontogenetic younger trees, and explants collected in spring had a substantially higher proportion of developed shoots. The genotype performance on the stabilised culture had substantial variation with several growth patterns. Clones had a consistent response in terms of the length of the main shoot among different media content. Our results suggest hyperhydration as a key limiting factor for the in vitro propagation of silver birch genotypes. The media with an optimal balance between multiplication and hyperhydration should be carefully selected to reduce the waste of resources. Yet, economic implications should be addressed with caution because even small differences in the multiplication rate between genotypes within a subculture result in the expansion in the number of produced seedlings per genotype in a year. Moreover, the multiplication capability should be viewed in the context of the rooting and acclimatisation of the seedlings and the field performance of the given genotypes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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