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Possibilities for reproduction and archiving resulting in a clone collection of a unique grey poplar (*Populus* × *canescens* Aiton Sm.) population in the Czech Republic

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Abstract
Floodplain forests as one of the most endangered ecosystems in Europe have recently been impacted by fungal pathogens *Phytophtora* sp. and *Hymenoscyphus fraxineus* (chalara). Grey poplar (*Populus* × *canescens* Aiton Sm.) can be used as one of well adapted tree species for the reforestation of withering stands of ash (*Fraxinus* sp.). A unique population of grey poplar (*Populus* × *canescens* Aiton Sm.) characterized by desirable phenotypic traits was used for this purpose. The gender distribution was asymmetric; out of 155 individuals, 113 were female. Out of 33 different genotypes determined in the population, 15 were used as a source of approved forest reproductive material. Vegetative reproduction methods (*ex situ* clonal reproduction by micropropagation and cuttings) were developed and used, to rapidly initiate the recovery of forest stands of grey poplar. In total, 940 explants were successfully micropropagated and adapted to natural conditions, to ensure a genetically diverse source of viable plants used for reforestation. Moreover, we used methodological procedures of micropropagation for setting up cryopreservation technique. With respect to long-term storage of valuable grey poplar genotypes, modulation of gene expression by cold hardening during cryopreservation revealed significant changes in a few candidate genes involved in plant cellular processes.

Key words: grey poplar (*Populus* × *canescens* Aiton Sm.); genetic diversity; clonal identity; *in vitro* biotechnology; reforestation; qPCR

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1. Introduction

Poplars (genus *Populus*) are progressively studied at the molecular biology, biotechnology, ecological and economic levels. They serve as a valuable source of carbon sequestration, bioremediation, nutrient cycling and biofiltration. Due to their rapid growth, modest genome size and extensive availability of expressed sequence tags, they have been used as experimental material for facile transformation and vegetative propagation (Brunner et al. 2004). Poplars belong to the *Salicaceae* family, which includes a total 582 records of *Populus* species along with more than 100 accepted names (Guleria et al. 2022). The diploid chromosome number is 2n = 38. Species in the genus *Populus* have been historically classified by morphological and reproductive characters into six sections: Abaso, Leuce (*Populus*), Aigeiros, Tacamahaca, Leucoides, and Turanga, based on their morphological traits and crossability (Eckenwalder 1996). However, using molecular markers and sequence methods, it was revealed that section Abaso is the oldest and most differentiated from the other sections (Cervera et al. 2005). Grey poplar (*Populus* × *canescens* Aiton Sm.) was originally described as a distinct species, but genetic marker-based and experimental studies revealed its hybrid origin that spontaneously resulted from the hybridization of *Populus alba* × *Populus tremula* (white poplar and aspen, respectively). Both poplars, originating from the Populus section, are characterized by much broader ranges of habitat (Eckenwalder 1996; Van Loo et al. 2008). While *P. tremula* L. is a widespread tree distributed in mountainous and upland habitats and mixed conifer forests as well as in nearly pure stands in riparian areas, *P. alba* L. is almost exclusively riparian in some parts of its range in Europe (Lexer et al. 2005). Grey poplar is mostly distributed at localities of common occurrence of both parent species, as it is characterized by similar ecological requirements as white poplar. However, it is

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more resistant to drought and tolerates more acidic soils. It grows naturally relatively rarely in floodplain forests and riparian shrubs. The overall distribution area extends from northern Spain through Central and Southern Europe to western Siberia, with preferential backcrossing with *P. alba* L. rather than with ecologically more distant *P. tremula* L. (Santos-del-Blanco et al. 2013). In the Czech Republic, grey poplar occurs as autochthonous in the Moravian valleys and in the Svratka and Odra valleys (Buriánek & Novotný 2016). Grey poplar has not yet been intentionally and purposefully used in forest regeneration due mainly to its limited occurrence and its more difficult determination in stands for the possible collection of reproductive material.

Effective reproduction of poplars can be reached by generative and vegetative propagation. In our study we focused on both methods of grey poplar reproduction involving sowing seeds, vegetative reproduction by cuttings and micropropagation technique. For sexual reproduction, *Populus* reaches maturity within 4–8 years in intensively managed plantations, in contrast to the 10–15 years required to reach maturity under favourable conditions in natural populations (Stanton & Villar 1996). Individual trees flower for 1–2 weeks, but the pollination period can exceed one or even two months. Seeds are produced in great numbers (> 25 million per tree per year), and their small size and cotton-like appendages facilitate their dispersal over large distances by wind and water. Seeds normally retain viability for 1–2 weeks in natural systems and germinate within 24 h under optimal conditions (DiFazio et al. 2011).

For several poplar species, clonal propagation is characteristic, but the extent and modes of vegetative propagation vary among different sections of the genus *Populus*. Interestingly, aspen clones can reach enormous levels of vegetative propagation, as described e.g., Kemperman & Barnes (1976) for *P. tremuloides* Michx.. The clone of American aspen consists of 47,000 ramets and covers an area of 43 ha, spreading mainly through the production of vegetative sprouts from adventitious buds on shallow lateral roots (Perala 1990).

The easiest method of poplar vegetative propagation is by cuttings. Cuttage is one of the oldest technologies for poplar breeding and clonal propagation (Zhao et al. 2014). Due to the increasing demand for poplar woods as a promising resource of renewable carbon for bioenergy and biofuel, clonal propagation by cuttings has been among the more efficient and flexible methods, as described by several authors (e.g. Morgenson 1992). Successful propagation by cutting strongly depends on the development of adventitious roots, which are affected by factors other than the genetic disposition of specific poplar species for their rooting ability. For example, *P. alba* L. cuttings fully rooted after 14 days, in contrast to the reduced rooting in *P. tomentosa* Carrière and the lack of root establishment in *P. davidiana* Dode (Chen et al. 1994). Therefore, the selection of suitable genotypes (clones) with high rooting ability from the genus *Populus* is very important to avoid low survival rates of cuttings and economic losses (Zhao et al. 2014).

Cultivation of tissue cultures is a method for the rapid vegetative propagation of poplars and could be effectively used to gain sufficient amounts of valuable genotype of selected plant species for its long-term storage under ultralow temperature, in a short time (Pokorná et al. 2020). To establish tissue cultures of poplars, axillary buds taken from either leafed or dormant branches are mostly used (Whitehead & Giles 1977) whereas individual components of medium need to be adapted even for different clones of one species (Whitehead & Giles 1977; Kutsokon et al. 2013).

The most innovative, affordable and useful biotechnological technique to conserve plant material without the risks of genetic modifications from a long-term perspective is cryopreservation (Benelli 2021). One of the most popular cryogenic procedures is vitrification (Sakai et al. 1990), a physiological process that avoids intracellular ice crystallization during ultrafreezing (–196 °C) by the transition of the aqueous solution of the cytosol into an amorphous glassy state. Using this process, plant tissues and cells can be protected from damage and remain viable during their long-term storage under ultralow temperature (–196 °C), as a positive role of cold hardening in plant recovery during precultivation was observed (Lambardi et al. 2000). Various cryopreservation protocols for different plant species and tissues, including poplars, have been developed (e.g., Lambardi et al. 2000; Vidyagina et al. 2021). The effects of cold hardening on physiological, metabolic and protein expression changes in aspen were observed by Jouve et al. (2000). While storage of microcuttings for more than 1 year at 10 °C limited growth, survival was unaffected, and multiplication ability was satisfactory, but the endogenous abscisic acid and proline contents substantially changed. Similarly, changes in gene expression profiles after 7 days of cold treatment were detected by Benedict et al. (2006) in poplar hybrids. Maestrini et al. (2009) showed that in white poplar treated with low temperature (4 °C) for 3–48 h, the highest percentage distributions of upregulated genes in leaves included those involved in metabolism and energy (26.5%) and stress and defence (10.5%).

Due to the high infectious pressure of the fungal pathogens *Phytophtora* sp. and *Hymenoscyphus fraxineus* (chalara) in withering stands of ash (*Fraxinus* sp.) in floodplain forests, which are among the most endangered ecosystems in Europe (Čížková 2007), there is an urgent need to select the best adaptive tree species for replacing the decaying ash stands in Czechia. Native poplar species are irreplaceable forest ecosystems and could serve as a valuable tree species with considerable potential to create new mixed healthy stands that could preserve functional and stable floodplain forest ecosystems. For this purpose, a unique, forestry-valuable, high-quality population of grey poplar characterized by desirable

phenotype traits located in floodplain forest in the South Moravia (Czechia) was used. In our study, we characterized the growth and phenotype traits of the poplar trees and selected the most valuable genotypes for reforestation. Characterization of a few genes in response to cold hardening before cryopreservation was also performed.

2. Material and methods

2.1. Plant material

A unique population of grey poplar is located in the Znojmo region (forest administration Znojmo, forest district Jaroslavice) near Dyjákovice village (South Moravia, Czech Republic) in a small floodplain forest complex Lužný forest with an area of 15 ha surrounded by an intensive agricultural landscape (Fig. 1). In the population, qualitative and quantitative traits were characterized in 155 individuals (marked as TPE1–TPE201) with a previous estimation of genetic diversity for all of them (Pokorná et al. 2018). In accordance with the requirements for the phenotypic quality of forest reproductive material sources, we searched for nonidentical individuals suitable for the establishment of a clone collection and simultaneously supporting the conservation of this unique population. Therefore, part of the inventory was a thorough selection of grey poplars from all age classes (6–9 classes) characterized by excellent health, straight shape, trunk length and excellent cleaning of the trunk. The sex of each registered individual was determined in the flowering season. Due to the expected occurrence of clonality in the stands, specific phenotypic traits possibly determining certain genotypes, e.g. branching angle, occurrence of strong branches in the crown or crown density, were also observed. For all selected plus trees, *in vitro* cultures were established (Žižková et al. 2017).

2.2. Morphological and phenotypic classification

Quality individuals of grey poplars were selected based on phenotypic characteristics used for plus tree selection according to Czech forestry regulations. Desirable characteristics were trunk cleaning (excellent or good), trunk shape (straight or slightly curved), length of crown (short or medium), and crown shape (oval or spherical). Further characteristics, such as prevailing thin branches in the crown and a branching angle of 45–60°, were included in the assessment. The dendrometric parameters tree height (h) and diameter (dbh) were measured. The occurrence of some traits depended on natural processes in stands, especially the high density of coppices during the first decades as well as the age of individuals. These factors were included in the complete investigation. The morphological traits of the leaves, bark, buds and flowers of grey poplar were thoroughly studied based on an existing mixture of the parental species *P. alba* L. and *P. tremula* L. with *P.* × *canescens* Aiton Sm. in the stands. Verified individuals were phenotypically classified according to the requirements of the forestry regulations, and a representative group of grey poplars previously genotyped by selected nuclear microsatellites (Pokorná et al. 2018) was subsequently used for establishing a clone collection.

Fig. 1. Sampling of grey poplar (*Populus* × *canescens* Aiton Sm.) individuals near Dyjákovice village (South Moravia, Czech Republic).

2.3. Generative propagation

Seeds were collected from branches of seven adult plus trees (TPE1, TPE12, TPE15, TPE22, TPE25, TPE61 and TPE62) verified as the identical genotype (Pokorná et al. 2018). In female clones were characterized qualitative and quantitative traits at the end of April 2016 and seeds were stored at 1–2 °C for short-term storage or at –18 °C for long-term storage. The germination rate of the seeds reached 90%. Seeds were sown following standards used for poplars on the surface of peat in QuickPot T60 planters in a greenhouse at the end of May 2016 (Čížková 2011) in a forest nursery (StromBarnet Ltd., Czech Republic). Finally, seedlings were transplanted into 1-litre pots after two weeks and planted under pest control conditions with regular foliar nutrition. Seedlings were suitable for reforestation after five months, with an average height of 0.8 m.

2.4. Vegetative propagation by cuttings

Rooting ability of grey poplar was tested on axial woody cuttings, originating from adult trees but due to their very low rooting ability we rejuvenilized plant material by *in vitro* technology and a one-year micropropagated plantlets were used for the classic cutting method. Seven genetically identical female clones (TPE18, TPE69, TPE77, TPE88, TPE122, TPE155 and TPE168) were tested. Dormant plant material was cut in the beginning of March and stored in a cold place at 2 °C. Cuttings 0.1 m in lenght were placed in wet substrate mixed as peat and perlite at a ratio 8 : 2 in a greenhouse at the end of March. Two alternatives, with and without 0.1% indole-3-butyric acid (IBA) stimulation, with 26 cuttings in three biological replications for each variant, were compared. Statistically significant differences of rooting ability between the untreated and treated plants with 0.1% IBA within each clone were estimated according to chi-square test, ($P < 0.05$).

2.5. Vegetative propagation by *in vitro* cultures

The collection of grey poplar explants was established from dormant buds of 155 selected individuals. The micropropagation of grey poplar and the rooting and acclimatization of explants were performed according to Malá et al. (2006) and Pokorná et al. (2017).

2.6. Cold hardening of grey poplar explants

An *in vitro* grown culture of grey poplar (*Populus* × *canescens* Aiton Sm.) TPE111 characterized with highly occuring genotype in our study population was established in enriched MS medium as previously described by Žižková et al. (2017). Explants were maintained at 24 °C with a 16 h/8 h light photoperiod with a light intensity of 30 µmol m^{-2} s^{-1} and periodically subcultured (every 4 weeks). *In vitro* cultures were divided into two groups (control and cold hardening) according to their cultivation temperatures. The control represented *in vitro* shoots cultivated at 24 °C while cold-hardened shoots were cultivated at 6 °C. Both groups of explants were cultivated for 6 weeks under the same photoperiod and light intensity as is mentioned above.

2.7. RNA extraction and gene expression analysis in grey poplar explants

Total RNA was extracted from homogenized grey poplar explants (control and cold hardening) individually (100 mg FW) using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Extracted RNA was quantified and checked for quality on a MaestroNano Pro spectrophotometer (MaestroGen, Las Vegas, NV, USA). Samples were treated with an RNase-Free DNase I Kit (Norgen Biotek) to remove genomic DNA contamination. Aliquots of RNA (2,000 ng) from at least three biological replicates were reverse-transcribed to cDNA with the Transcriptor First Strand cDNA Synthesis Kit (Roche, Switzerland) and oligo-dT_{18} primers (IDT Integrated DNA Technologies) in a total volume of 13 µl according to the manufacturer's instructions. Diluted cDNA samples at a volume of 2.5 µl each were used for qRT-PCRs containing 5 µl of FastStart Essential DNA Green Master (Roche, Switzerland), 2.5 µM of each primer and 1.5 µl of ultra-purified water. qRT-PCR analyses were performed to evaluate the expression of four target genes (*CDC2 – cell cycle division 2, Chitin2 – endochitinase 2, GST – glutathione S-transferase* and *PhotII – photosystem II*) and two reference genes, *Actin2* and *ubiquitin 7* (*UBQ7*), as reported previously for the poplar species Komárková et al. (2020) and Pettengill et al. (2012). Each of the cDNA samples was run in at least two technical replications on a LightCycler 96 (Roche, Switzerland) with the following cycling conditions: preincubation for 120 s at 95 °C, followed by amplification for 45 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s. The melting curve was assessed at 95 °C for 10 s, followed by 65 °C for 60 s and 97 °C for 1 s. Quantification of gene expression was performed according to the ΔCt method with Ct values normalized for *Actin2* and *UBQ7* (Vandesompele et al. 2002). Statistically significant differences between the controls and the cold-hardened samples were estimated according to unpaired Student's *t* test, ($P < 0.05$).

3. Results

3.1. Phenotypic traits of grey poplar individuals in the Lužný forest

In a unique population of grey poplar (Fig. 1) characterized by desirable phenotypic traits (Fig. 2), we determined several quantitative parameters according to Novotný et al. (2021) and Nanson (2001) to describe 155 individuals (120 females and 35 males) in more details. Among grey poplars of female sex, the phenotype of selected plus trees prevailed in 113 individuals (determined as genetically uniform by Pokorná et al. 2018) characterized by a high density of thicker branches in narrow oval crowns with a branching angle of 30° (e.g., Fig. 2A–2F, Table 1); male clones were not very different, with almost all trees characterized by very thin round crowns and straight stems with a few lateral branches (Fig. 2G and 2H, Table 1). The other individuals of female sex, e.g. TPE107, had round, short, dense crown with fine branchiness, and TPE108 had repeated fork branchiness in long crown, whereas TPE151 was very similar to male phenotypes (Fig. 2D–2F, Table 1). Phenotypes of selected plus trees, such as long, straight, naturally pruned stems, regular short crowns, prevailing thin branches and branching angles of 45–60°, were found in several grey poplar individuals, which were subsequently selected for further vegetative and generative reproduction.

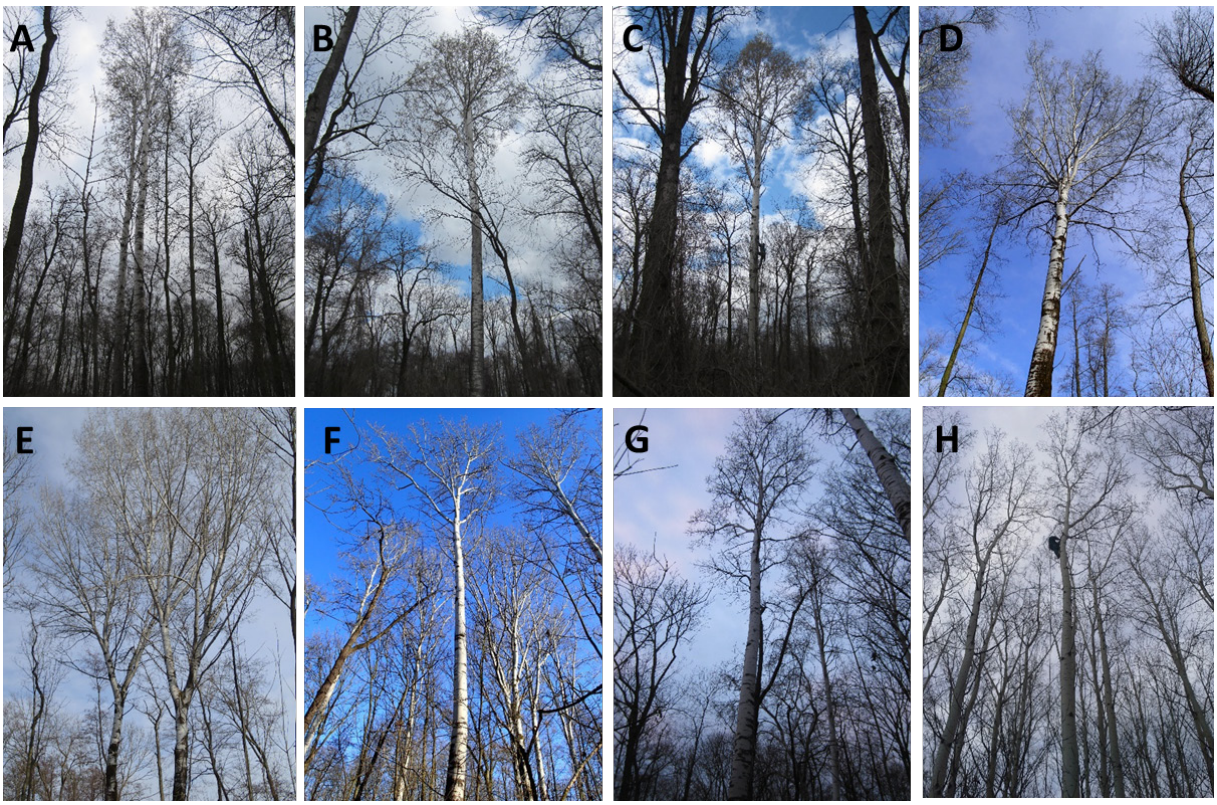

Fig. 2. Individuals of grey poplar (*Populus* × *canescens* Aitom. Sm.) located in Dyjákovice village, Czech Republic. Grey poplars of female sex, TPE133 (2A), TPE135 (2B), TPE163 (2C), TPE107 (2D), TPE108 (2E), TPE151 (2F) and grey poplars of male sex, TPE149 (2G) and TPE194 (2H).

Table 1. Phenotypic classification of selected grey poplar (*Populus* × *canescens* Aitom. Sm.) individuals in the Lužný forest. Trunk shape: 1 – straight, 2 – slightly curved, 3 – strongly curved; trunk cleaning: 1 – excellent, 2 – good, 3 – poorly; crown shape: 1 – oval, 2 – egg shape, 3 – conical, 4 – spindle, 5 – reverse oval, 6 – umbrella shape, 7 – cylindrical, 8 – spherical, 9 – flag shape; crown length: 1 – short, 2 – medium, 3 – long.

Figure (clone)	Sex	Height (m)	Dbh (cm)	Trunk shape	Trunk cleaning	Crown shape	Crown length
2D (TPE107)	f	31	70	1	1	8	1
2E (TPE108)	f	28.5	70	1	1	8	1
2A (TPE133)	f	26	65	1	1	8	1
2B (TPE135)	f	27	67	1	1	8	1
2C (TPE163)	f	25	58	1	1	1	1
2F (TPE151)	f	24	55	1	2	8	1
2G (TPE149)	m	23	48	1	1	1	1
2H (TPE194)	m	23	36	1	1	8	1

Grey poplar clones selected for establishing a clone collection (Table 4) in the category Qualified are genetically distinct, which has been confirmed by analyses using 14 polymorphic SSR markers in previous study (Pokorná et al. 2018).

3.2. Generative propagation of grey poplar

Seeds from genetically identical grey poplars (TPE1, TPE12, TPE15, TPE22, TPE25, TPE61 and TPE62) of female sex with desirable qualitative as well as quantitative traits were used for the generative reproduction method. Overall, more than 10,000 seedlings of grey poplars (Fig. 3) were obtained in the forest nursery (StromBarnet Ltd., Czech Republic).

Fig. 3. Generative propagation of grey poplar (*Populus* × *canescens* Aiton Sm.) selected genotypes (female sex) in the forest nursery (StromBarnet Ltd., Czech Republic).

Seedlings were deemed suitable for reforestation after five months of cultivation in pots with regular foliar nutrition and pest control. Finally, grey poplar seedlings with an average height of 0.8 m were used as an alternative tree species for reforestation after ash dieback in several localities (Table 2) under the control of Forest Service Židlochovice and Znojmo.

Table 2. Grey poplar seedlings used for reforestation at several localities in the Czech Republic.

Year	Number of seedlings	Forest service	Location
2017	1,100	Židlochovice	Valtice
	2,000	Židlochovice	Velký Dvůr
	133	Znojmo	Jaroslavice
	600	Znojmo	Višňová

3.3. Vegetative propagation of grey poplar by cuttings

To obtain young plants of selected grey poplar plus trees, we used axial woody cuttings for grey poplar reproduction. We found a very low root formation ability in cuttings originating from grey poplar adult trees; therefore, we used younger plant materials for vegetative propagation. In cuttings from genetically identical female individuals (TPE18, TPE69, TPE77, TPE88, TPE122, TPE155 and TPE168) transferred into the substrate, we observed the formation of sprouting buds (after approximately 4 weeks) followed by growing shoots reaching up to 10–12 cm over 3 weeks (Fig. 4A). After treating cuttings with 0.1% IBA, we evaluated the effect of synthetic auxin on root formation (Fig. 4B).

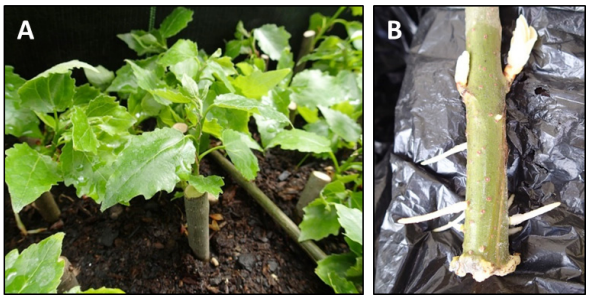

Fig. 4. Vegetative reproduction of grey poplar (*Populus* × *canescens* Aiton Sm.) by axial woody cuttings. Cutting with growing shoot (A) and root formation on cutting (B).

Our results show that the rooting ability of grey poplar cuttings, even of identical genotypes, substantially varied in both untreated and treated cuttings (Fig. 5). We observed better root formation in the TPE77 and TPE122 clones in the untreated cuttings than in the TPE18, TPE69, TPE88 and TPE168 clones treated with 0.1% IBA. The effect of synthetic auxin on rooting ability in grey poplar cuttings reached 56.4% to 100%, whereas we detected maximum rooting ability (100%) in clones TPE69 and TPE88 (Fig. 5). Interestingly, no effect of 0.1% IBA was observed in clone TPE155 (Fig. 5). Although we observed different effects on root formation in untreated as well as treated plants with 0.1% IBA, no statistically significant differences within each clone of grey poplar (identical clones of female sex) were found.

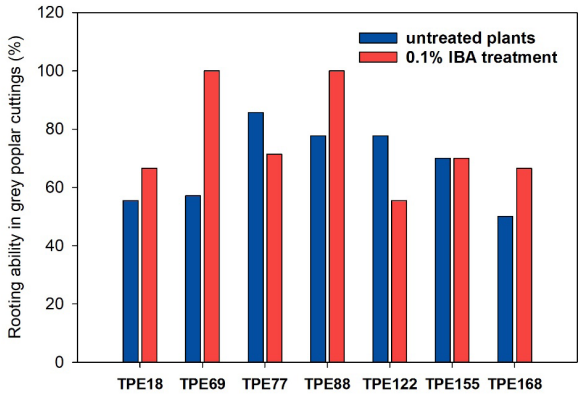

Fig. 5. Rooting ability in grey poplar (*Populus* × *canescens* Aiton Sm.) axial woody cuttings of the identical genotype of female sex.

3.4. *Ex situ* preservation of grey poplar clones by use of *in vitro* culture

To preserve the unique phenotypic traits of the grey poplar population and ensure the efficient reproduction of selected plus trees for subsequent plantings, a method for effective micropropagation was developed and used (Pokorná et al. 2017; Žižková et al. 2017). In total, we tested six different genotypes of female sex and twenty seven different genotypes of male sex for multiplication ability and ability to establish roots. Comparison of female and male genotypes revealed more successful induction of organogenesis, multiplication and root formation ability in female than in male genotypes. Reproductive success in females reached 83.3% (detected in five different genotypes) opposite to much lower multiplication ability in males reaching 25.9% (detected only in seven different genotypes; Table 3).

For the other selected grey poplar individuals, based on the frequency of microcut formation and their rooting ability, we recorded considerable differences during the cultivation period (Table 3). The most adapted rooting microcuts to semisterile conditions were obtained from mostly identical clones of female sex, comprising a total of 765 plantable plants (Table 3). In total, we obtained 940 plantlets that were subsequently transferred into the greenhouse and, after two weeks, into a half-shade flower bed. Healthy and appropriately developing plants were moved into larger pots and ultimately used (430 of them) for reforestation at the locality in Znojmo, forest area Lechovice (CZ).

3.5. Establishing clone collection

To preserve desirable qualitative traits and support genetic diversity in the prevailing clonal population of grey poplars, 15 genotypes of both males and females (10 and 5, respectively) were selected as plus trees based on their phenotypic traits and genetic analyses and were approved as sources of reproductive material in the category Qualified for the collection of both vegetative and reproductive material (Table 4). Seeds from these different genotypes with selected plus tree parameters were deemed suitable for generative propagation, especially by Lesy ČR, s. p. (Czech State Forest) as an owner. In addition, all 15 selected genotypes were used for establishing clone collection in the category Qualified, which is located in Kostelany nad Moravou (Czech Republic, Fig. 6) and represents plants propagated by vegetative methods (micropropagation and grafting; Table 4). Based on our previous experiences with micropropagation of different grey poplar genotypes of both sexes

Table 3. Number of grey poplar (*Populus* × *canescens* Aiton Sm.) plants propagated *in vitro*. Grey poplar explants marked with the same colour are identical clones and without colour are marked genetically nonidentical individuals.

Sex	Genotype	Number of plantable plants	Sex	Genotype	Number of plantable plants	Sex	Genotype	Number of plantable plants
female	TPE 3	23	female	TPE 84	7	female	TPE 166	20
	TPE 7	8		TPE 85	13		TPE 168	26
	TPE 16	13		TPE 86	15		TPE 169	1
	TPE 18	16		TPE 88	31		TPE 170	14
	TPE 19	19		TPE 89	14		TPE 174	8
	TPE 22	9		TPE 109	8		TPE 187	25
	TPE 23	5		TPE 111	17		TPE 190	15
	TPE 33	18		TPE 113	23		TPE 196	20
	TPE 47	11		TPE 120	29		TPE107	23
	TPE 48	12		TPE 122	36		TPE108	5
	TPE 52	10		TPE 124	26		TPE199	4
	TPE 56	7		TPE 133	5		TPE200	16
	TPE 67	15		TPE 135	20	male	TPE162	6
	TPE 68	3		TPE 136	19		TPE191	7
	TPE 69	18		TPE 137	35		TPE125	24
	TPE 71	4		TPE 155	24		TPE192	19
	TPE 75	6		TPE 157	9		TPE167	19
	TPE 77	17		TPE 159	29		TPE186	6
	TPE 78	21		TPE 163	11		TPE188	21
	TPE 83	7		TPE 165	23		TPE193	25

Table 4. Phenotypic classification of clonal components used for grey poplar (*Populus* × *canescens* Aitom. Sm.) clone collection. Trunk shape: 1 – straight, 2 – slightly curved, 3 – strongly curved; trunk cleaning: 1 – excellent, 2 – good, 3 – poorly; crown shape: 1 – oval, 2 – egg shape, 3 – conical, 4 – spindle, 5 – reverse oval, 6 – umbrella shape, 7 – cylindrical, 8 – spherical, 9 – flag shape; crown length: 1 – short, 2 – medium, 3 – long.

Clone	Sex	Height (m)	DBH (cm)	Trunk shape	Trunk cleaning	Crown shape	Crown length
TPE101	m	25	52	2	1	8	1
TPE102	m	26	47	2	1	8	1
TPE103	m	28.5	55	1	1	1	1
TPE107	f	31	70	1	1	8	1
TPE108	f	28.5	70	1	1	8	2
TPE127	m	25	39	1	1	6	1
TPE130	m	25	37	1	1	5	1
TPE132	m	28	55	2	1	8	2
TPE150	m	25	58	1	1	8	1
TPE151	f	24	55	1	2	8	1
TPE164	m	26	66	1	1	8	1
TPE169	f	24	44	1	1	1	1
TPE186	m	30	53	2	1	1	1
TPE194	m	23	36	1	1	8	1
TPE199	f	35	103	1	1	8	1

(Table 3), we used younger plant material from grey poplar clones for induction of organogenesis that was finally much more successful. This clone collection will serve as a stool bed for the vegetative reproduction of grey poplar.

Fig. 6. Clone collection established in Kostelany nad Moravou (Czech Republic).

3.6. Gene expression changes in grey poplar explants precultured at low temperature during cryopreservation

The most prevailing grey poplar genotype with phenotypic traits of plus trees from Dyjákovice village was previously selected for long-term storage at –196 °C. To characterize the physiological state of plants at the molecular level, we focused on genes associated with cellular processes and followed their responses to cold hardening during grey poplar explant acclimatization. Our results showed that cold hardening significantly downregulated *CDC2* and *Phot II*, genes associated with cell cycle progression, in comparison to control conditions. In contrast, *Chitin2*, which is involved in carbohydrate metabolism and polysaccharide degradation, and *GST*, a gene induced by stress conditions, were upregulated 19- and 3-fold, respectively (Fig. 7). Our preliminary data suggest that cold hardening significantly affects plant physiological processes at the molecular level.

4. Discussion

Floodplain forests, as one of the most endangered ecosystems in Europe, have recently been impacted by the fungal pathogens *Phytophtora* sp. and *Hymenoscyphus fraxineus* (chalara) in withering stands of ash (*Fraxinus* sp.). Grey poplar (*Populus* × *canescens* Aiton Sm.) can be used as a well-adapted tree species for the reforestation of floodplain forest ecosystems (Brown et al. 1997). A unique population of grey poplar characterized by desirable phenotypic traits was investigated as a potential source of forest reproductive material. We characterized the phenotypic traits of both male and female individuals (35 and 120, respectively). The observed phenotypic variation in selected plus trees (Fig. 2, Table 1) could be strongly influenced by genetic and environmental factors, as reported previously Smith et al. (2011) for quaking aspen (*Populus tremuloides* Michx.) clones. Similarly, Xue et al. (2019) demonstrated biological adaptation by modelling general forms of phenotypic responses to environmental conditions and described how traits of organisms or behaviours could depend on the environment. We

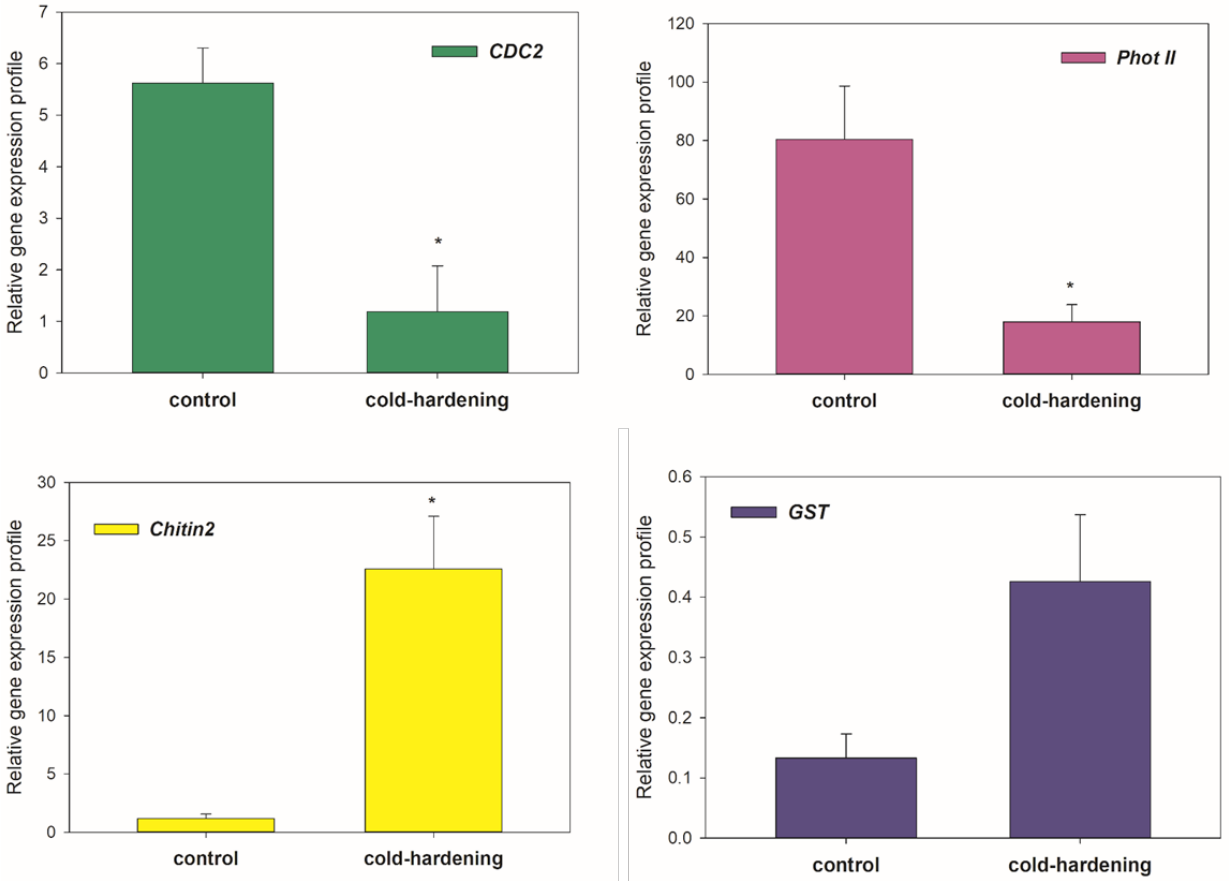

Fig. 7. Modulation of relative gene expression profiles during cold hardening treatment (6 weeks) in grey poplar (*Populus* × *canescens* Aiton Sm.) explants compared to the control. Data are expressed as the mean value ± SD (n = 4) of the transcript levels of each target gene. Asterisks indicate significantly differentially regulated transcripts compared to control conditions (Student's *t* test, $P < 0.05$).

characterized the variability in trunk shape and cleaning among genetically uniform grey poplar individuals (previously published Pokorná et al. 2018), which may be related to site quality, as published Barnes (1969).

The method of plant tissue *in vitro* culture, which enables a fast and efficient propagation of poplars (Pokorná et al. 2017; Žižková et al. 2017) and was used for preservation of 155 selected plus trees of grey poplar individuals revealed differences in the ability to proliferate and induce organogenesis from dormant buds (Table 3). Currently, several protocols for micropropagation and regeneration of *Populus* species are practised and it is well known that the success of micropropagation is genotype-specific, even within the same species (Douglas 1984; Confalonieri et al. 2003). According to ability to form roots in grey poplar explants, we vegetatively propagated by micropropagation and grafting, 15 selected genotypes (Table 4), both male and female, to serve as a source of qualified reproductive material for forest owners. Plantlets that were fully acclimated in greenhouse and natural conditions were subsequently transferred to the Kostelany nad Moravou locality (Fig. 6). Consistently with our aim of study, Keserű et al. (2015) preserved selected plus trees of poplar clones and provided a published selective breeding micropropagation method for poplars. Similarly, several authors demonstrated a successful production of viable plants from dormant buds of diverse forest tree species through *in vitro* techniques with their subsequent planting in demonstration plots (Cvrčková et al. 2007; Dostál et al. 2011, 2016; Komárková et al. 2022). Growth and morphological parameters were followed by Cvrčková et al. (2007) and Dostál et al. (2011 and 2016) in demonstration plots, who revealed that vegetatively propagated plantlets and generatively propagated plants have comparable characteristics, suggesting that micropropagation of forest tree species is a very effective method for ensuring high-quality reproductive materials for plantings. In addition, we showed that vegetative propagation by axial woody cuttings (Fig. 4 and Fig. 5) is a practical method of reproduction of selected grey poplar genotypes recommended for forest nurseries, which can maintain a poplar stool bed as a source of fresh cuttings. On the other hand, generative reproduction originating from grey poplar seeds was a very effective method for large-scale production of seedlings (Fig. 3, Table 2), but differences in the genetic structure of offspring due to the splitting of hybrid parents and pollination conditions must be taken into account.

Due to reductions in genetic diversity and genetic structure in poplar populations (Palancean et al. 2018) for white poplar, it is necessary to preserve selected plus tree genotypes from a long-term perspective. Positive effects of cold hardening (4 °C) during the precultivation of white poplar shoot tips on successful cryopreservation by one-step vitrification were published by Lambardi et al. (2000). Similarly, Pokorná et al. (2020) observed positive effects of cold hardening during pretreatment conditions on the postthaw recovery of grey poplar explants. Although low temperature is one of the environmental stresses affecting plant productivity (cold triggers cell death by cytoplasmic dehydration and ice formation in the cell wall), plants acclimated to low temperature are more tolerant to freezing due to the activation of several protective mechanisms (Lissarre et al. 2010). During cold acclimatization in plants, numerous physiological, biochemical and molecular changes occur that are associated with transcriptome regulation. In our preliminary data, we observed the significant regulation of genes involved in plant fundamental physiological processes such as photosynthesis, cell cycle regulation, defence systems and stress. Similarly, upregulation of *Chitin2* and *GST* in response to stress conditions, such as low temperature or exposure to heavy metals, was observed by Komárková et al. (2020) in grey poplar shoots and roots after 2 days of 10 µM Cd treatment. Although the gene expression of *Phot II* was not altered after 2 days of 10 µM Cd treatment, we observed significant downregulation by cold hardening in our samples (Fig. 7). Wu et al. (2022) found that silage corn seedlings cultivated for 5 days at 5 °C exhibited the highest morphological, physiological and biochemical changes, such as slow growth. We suggest that the slow growth detected in our samples (data not shown) caused by cold hardening is associated with significant downregulation of the *CDC2* and *PhotII* genes (Fig. 7), both of which regulate plant cellular processes.

5. Conclusions

Native European species of poplars are an important component of floodplain forests. Forest management has recently find new solutions for reforestation to increase biodiversity and stability of forest ecosystems with high resilience to pathogens. Grey poplar (*Populus* × *canescens* Aiton Sm.) can be used as a well-adapted tree species for the reforestation of floodplain forest ecosystems. In a unique population of grey poplar located in Dyjákovice village (South Moravia, Czech Republic), the growth, phenotypic traits and level of genetic diversity were previously studied (Pokorná et al. 2018). The set of 15 clones was approved as plus trees and for establishing a clone collection in the category Qualified. Generative and vegetative propagation methods were tested and modified to forest nursery methods in the Czech Republic, and thousands of plants were used for reforestation to start practical plantations of neglected tree species by forest owners.

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References

Barnes, B. V., 1969: Natural variation and delineation of clones of *Populus tremuloides* and *P. grandidentata* in northern Lower Michigan. Silvae Genetica, 18:130–142.

Benedict, C., Skinner, J. S., Meng, R., Chang, Y., Bhalerao, R., Huner, N. P. et al., 2006: The CBF1-dependent low temperature signalling pathway, regulon and increase in freeze tolerance are conserved in *Populus* spp. Plant Cell Environment, 29:1259–1272.

Benelli, C., 2021: Plant Cryopreservation: A look at the present and the future. Plants, 10:2744.

Brown, A. G., Harper, D., Peterken, G. F., 1997: European floodplain forests: Structure, functioning and management. Global Ecology and Biogeography Letters, 6:169–178.

Brunner, A. M., Busov, V. B., Strauss, S. H., 2004: Poplar genome sequence: functional genomics in an ecologically dominant plant species. Trends in Plant Science, 9:49–56.

Buriánek, V., Novotný, P., 2016: Metodická příručka k určování domácích druhů topolů. Lesnický průvodce č. 11, Certifikovaná metodika. Strnady, Výzkumný ústav lesního hospodářství a myslivosti, v. v. i., 35 p. (In Czech).

Cervera, M. T., Storme, V., Soto, A., Ivens, B., Van Montagu, M., Rajora, O. P. et al., 2005: Intraspecific and interspecific genetic and phylogenetic relationships in the genus *Populus* based on AFLP markers. Theoretical and Applied Genetics, 111:1440–1456.

Chen, X., Gao, H., Wang, S., 1994: Studies on endogenous hormone levels in cuttings of three poplar species during rooting process. Scientia Silvae Sinicae, 30:1–7.

Čížková, L., 2007: Možnosti ochrany genových zdrojů domácích druhů topolů, in: Ohrožené dřeviny ČR. In: Dreslerová, J., Packová, P. (eds.): Ohrožené dřeviny ČR. Geobiocenologické spisy, sv. č. 12, Sborník příspěvků z konference, 8. – 9. 2. 2007, Brno, p. 57–60. (In Czech).

Čížková, L., 2011: Generativní množení topolů. Lesnický průvodce č. 5. Certifikovaná metodika. Strnady, Výzkumný ústav lesního hospodářství a myslivosti, v. v. i., 19 p. (In Czech).

Confalonieri, M., Balestrazzi, A., Bisoffi, S., Carbonera, D., 2003: *In vitro* culture and genetic engineering of *Populus* spp.: synergy for forest tree improvement. Plant Cell, Tissue and Organ Culture, 72:109–138.

Cvrčková, H., Malá, J., Máchová, P., Novotný, P., 2007: Růst a vývoj výpěstků *in vitro* třešně ptačí (*Prunus avium* L.) a dubu letního (*Quercus robur* L.) na demonstračních plochách. Zprávy lesnického výzkumu, 52:123–131.

DiFazio, S. P., Slavov, G. T., Chandrashekhar, P. J., 2011: *Populus*: A premier pioneer system for plant genomics. In: Chandrashekhar, P. J., DiFazio, S. P., Chittaranjan, K. (eds.): Genetics, genomics and breeding of poplar. USA, CRC Press, p. 1–28.

Dostál, J., Novotný, P., Čáp, J., 2016: Comparison of growth development of micropropagated and generatively reproduced wild cherry (*Prunus avium* L.) on the Polná demonstration plot (Czech Republic) up to the age of 15 years. Journal of Forest Science, 62:204–210.

Dostál, J., Cvrčková, H., Novotný, P., 2011: Porovnání růstových schopností *in vitro* sazenic různých druhů dřevin na ploše „Polná II". Zprávy lesnického výzkumu, 56:9–14.

Douglas, G. C., 1984: Formation of Adventitious Buds in Stem Internodes of *Populus* spp. Cultured *in vitro* on basal medium: Influence of endogenous properties of explants. Journal of Plant Physiology, 116:313–321.

Eckenwalder, J. E., 1996: Systematics and evolution of Populus. In: Stettler, R. F., Bradshaw, H. D., Heilman, P. E., Hinckley, T. M. (eds.): Biology of *Populus* and its implications for management and conservation. Ottawa, NRC Research Press, pp. 7–32.

Guleria, I., Kumari, A., Lacaille-Dubois, M. A., Nishant, Kumar, V., Saini, A. K. et al., 2022: A review on the genus *Populus*: A potential source of biologically active compounds. Phytochemistry Reviews, 21:987–1046.

Jouve, L., Franck, T., Gaspars, T., Cattivelli, L., Hausman, J. F., 2000: Poplar acclimation to cold during *in vitro* conservation at low non-freezing temperature: metabolic and proteic changes, Journal of Plant Physiology, 157:117–123.

Kemperman, J. A., Barnes, B. V., 1976: Clone size in American aspens. Canadian Journal of Botany, 54:2603–2607.

Keserű, Z., Balla, I., Antal, B., Rédei, K., 2015: Micropropagation of Leuce-poplars and evaluation of their development under sandy site conditions in Hungary. Acta Silvatica et Lignaria Hungary, 11:139–152.

Komárková, M., Chromý, J., Pokorná, E., Soudek, P., Máchová, P., 2020: Physiological and transcriptomic response of grey poplar (*Populus* × *canescens* Aiton Sm.) to cadmium stress. Plants, 9:1485.

Komárková, M., Cvrčková, H., Dostál, J., Buriánek, V., Máchová, P., 2022: Long-term observation of *in vitro*-derived *Malus Sylvestris* [L.] Mill., the path from the bud to the tree. In: Küden, A. B. (ed.): Apple cultivation – recent advances. London. IntechOpen. Available at: https://doi.org/10.5772/intechopen.108359.

Kutsokon, N., Libantova, J., Rudas, V., Rashydov, N., Grodzinsky, D., Ďurechová, D., 2013: Advancing protocols for poplars *in vitro* propagation, regeneration and selection of transformants. Journal of Microbiology Biotechnology & Food Sciences, 2:1447–1454.

Lambardi, M., Fabbri, A., Caccavale, A., 2000: Cryopreservation of white poplar (*Populus alba* L.) by vitrification of *in vitro*-grown shoot tips. Plant Cell Reports, 19:213–218.

Lexer, C., Fay, M. F., Joseph, J. A., Nica, M. S., Heinze, B., 2005: Barrier to gene flow between two ecologically divergent *Populus* species, *P. alba* (white poplar) and *P. tremula* (European aspen): the role of ecology and life history in gene introgression: barrier to gene flow in *Populus*. Molecular Ecology, 14:1045–1057.

Lissarre, M., Ohta, M., Sato, A., Miura, K., 2010: Cold-responsive gene regulation during cold acclimation in plants. Plant Signaling and Behavior, 5:948–52.

Maestrini, P., Cavallini, A., Rizzo, M., Giordani, T., Bernardi, R., Durante, M. et al., 2009: Isolation and expression analysis of low temperature-induced genes in white poplar (*Populus alba*). Journal of Plant Physiology, 166:1544–1556.

Malá, J., Máchová, P., Cvrčková, H., Čížková, L., 2006: Aspen micropropagation: use for phytoremediation of soils. Journal of Forest Science, 52:101–107.

Morgenson, G., 1992: Vegetative propagation of poplar and willow. In: Landis, T. D. (ed.): Proceedings, Intermountain Forest Nursery Association. Utah, U. S. Department of Agriculture, Forest Service, Rocky Mountain Forest and Range Experiment Station, p. 84–87.

Nanson, A., 2021: The new OECD scheme for the certification of forest reproductive materials. Silvae Genetica, 50:181–187.

Novotný, P., Buriánek, V., Frýdl, J., Kaňák, J., Čížková, L., Fulín, M. et al., 2021: Metodické postupy a kritéria pro uznávání a zabezpečení zdrojů reprodukčního materiálu lesních dřevin. Lesnický průvodce č. 5. Certifikovaná metodika. Strnady, Výzkumný ústav lesního hospodářství a myslivosti, v. v. i., 190 p. (In Czech).

Palancean, I., Alba, N., Sabatti, M., de Vries, S. M., 2018: EUFORGEN Technical guidelines for genetic conservation and use for white poplar (*Populus alba*). European Forest Genetic Resources Programme (EUFORGEN), European Forest Institute, 6 p.

Perala, D., 1990: *Populus tremuloides* Michx. quaking aspen. In: Burns, R. M., Honkala, B. H., (eds): Silvics of North America: Volume 2. Hardwoods. Agriculture Handbook 654. Washington, DC, U. S. Department of Agriculture, Forest Service, p. 555–569.

Pettengill, E. A., Parmentier-Line, C., Coleman, G. D., 2012: Evaluation of qPCR reference genes in two genotypes of *Populus* for use in photoperiod and low-temperature studies. BMC Research Notes, 5:366.

Pokorná, E., Buriánek, V., Máchová, P., Dostál, J., Komárková, M., 2017: Nové poznatky o reprodukci topolu šedého v *in vitro* podmínkách. Zprávy lesnického výzkumu, 62:279–287. (In Czech).

Pokorná, E., Čížková, L., Máchová, P., Cvrčková, H., Buriánek, V., Komárková, M. et al., 2018: Charakterizace genetické variability lokální populace topolu šedého (*Populus* × *canescens* Aiton Sm.) s využitím SSR markerů a fenotypového hodnocení. Zprávy lesnického výzkumu, 63:281–289. (In Czech).

Pokorná, E., Faltus, M., Máchová, P., Zámečník, J., Fulín, M., 2020: Grey poplar explant acclimation to improve the dehydration tolerance and cryopreservation. Biologia Plantarum, 64:119–128.

Sakai, A., Kobayashi, S., Oiyama, I., 1990: Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var. *brasiliensis* Tanaka) by vitrification. Plant Cell Reports, 9:30–33.

Santos-del-Blanco, L., de-Lucas, A. I., González-Martínez, S. C., Sierra-de-Grado, R., Hidalgo, E., 2013: Extensive clonal assemblies in *Populus alba* and *Populus* × *canescens* from the Iberian Peninsula. Tree Genetics & Genomes, 9:499–510.

Smith, E. A., Collette, S. B., Boynton, T. A., Lillrose, T., Stevens, M. R., Bekker, M. F. et al., 2011: Developmental contributions to phenotypic variation in functional leaf traits within quaking aspen clones. Tree Physiology, 31:68–77.

Stanton, B., Villar, M., n.d. 1996: Controlled reproduction of Populus. In: Stettler, R. F., Bradshaw, H. D. Jr., Heilman, P. E., Hinckley, T. M. (eds): Biology of *Populus* and its implications for management and conservation. Ottawa, Ontario, Canada. NRC Research Press, p. 113–138.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. et al., 2002: Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology, 3: research0034.1–0034.11.

Van Loo, M., Joseph, J. A., Heinze, B., Fay, M. F., Lexer, C., 2008: Clonality and spatial genetic structure in *Populus* × *canescens* and its sympatric backcross parent P. alba in a Central European hybrid zone. New Phytologist, 177:506–516.

Vidyagina, E. O., Kharchenko, N. N., Shestibratov, K. A., 2021: Efficient cryopreservation of *Populus tremula* by *in vitro*-grown axillary buds and genetic stability of recovered plants. Plants, 10:77.

Whitehead, H. C. M., Giles, K. L., 1977: Rapid propagation of poplars by tissue culture methods. New Zealand of Forestry Science, 7:40–3.

Wu, J., Nadeem, M., Galagedara, L., Thomas, R., Cheema, M., 2022: Effects of chilling stress on morphological, physiological, and biochemical attributes of silage corn genotypes during seedling establishment. Plants, 11:1217.

Xue, B., Sartori, P., Leibler, S., 2019: Environment-to-phenotype mapping and adaptation strategies in varying environments. Proceedings of the National Academy of Sciences of America, 116:13847–13855.

Zhao, X., Zheng, H., Li, S., Yang, C., Jiang, J., Liu, G., 2014: The rooting of poplar cuttings: a review. New Forests, 45:21–34.

Žižková, E., Komárková, M., Máchová, P., Cvrčková, H., 2017: Metoda rychlé regenerace topolu šedého (*Populus* × *canescens* Aiton Sm.) s využitím *in vitro* organogeneze. Lesnický průvodce č. 5. Certifikovaná metodika. Strnady, Výzkumný ústav lesního hospodářství a myslivosti, v. v. i., 20 p. (In Czech).