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Grey poplar explant acclimation to improve the dehydration tolerance and cryopreservation

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Abstract

Cryostorage techniques have been developed to preserve the most valuable genotype from an endangered native population of *Populus × canescens* Aiton Sm. (grey poplar), which is located in the floodplain forest in the South Moravia region of the Czech Republic and which is difficult to propagate using cuttings. The prevailing genotype with valuable traits was selected by the simple sequence repeats method. This genotype was used to determine the most effective pre-cultivation conditions (cold hardening, and cold hardening combine with osmotic treatment) on dehydration tolerance and post-thaw recovery of the grey poplar shoot tips. The pre-cultivation and application of a modified plant vitrification solution 3 (PVS3) considerable reduced the freezable water content in shoot tips. Evaluation after eight weeks of regrowth revealed that simultaneous effect of cold and osmotic pre-treatments and application of PVS3 for 120 min enabled 93.3 ± 5.8 % recovery. The results emphasize the importance of the pre-cultivation conditions, which significantly improved the post-thaw recovery of the grey poplar explants.

Additional key words: apical segments, cold hardening, cryopreservation, *Populus × canescens*, SSR analysis, vitrification solution.

Introduction

Populus × canescens Aiton Sm. (grey poplar), a spontaneous hybrid of *Populus alba* L. and *Populus tremula* L., belongs among the woody plant species characterized by a fast growth rate with a relatively low nutrient demand and wide distribution in the river basins (Úředníček *et al.* 2009). Generally, *Populus* species have been previously utilized as a source of pulp and wood products, but recently, an interest in poplar significantly arose mainly due to the short rotation forestry regimes leading to production chips or pelletized wood fuel or feedstock for lignocellulosic bioethanol outputs (Guo *et al.* 2014). In the South Moravia region of the Czech Republic, a unique population of grey poplar specified by valuable traits, such as a narrow crown and a straight continuous trunk with natural pruning, has been determined in a floodplain forest (Buriánek and Novotný 2016). Based on a high interest to preserve the valuable phenotypic traits of grey poplars characterized by relatively high age and low effectivity of classical vegetative propagation by

cutting or grafting, cryopreservation is promissive method with long-term perspective. For experimental work, the most abundant genotype in the population based on determination of genetic traits was selected, which is very important for future strategy in forest management.

The preservation of viable cells, tissues, and organs in liquid nitrogen (LN, -196° C) represents a long-term storage method mainly used for plant genetic resources, which cannot be maintained *via* conventional preservation techniques, similar to recalcitrant seeds, vegetatively propagated specific cultivars or unique ornamental genotypes (Benson 1999, Rynänen 1999, Kacmarczyk *et al.* 2012). The cryopreservation has also been applied for rare and endangered species such as tropical forest trees or clonally propagated trees (Reed 2001, Wilkinson *et al.* 2003, Pritchard *et al.* 2014). There are several approaches and protocols for the cryopreservation of individual plant tissues in LN. In general, plant material with a high amount of cell water and thus endangered by intracellular water crystallization (buds, shoot tips, nodal segments, embryos, calli, and cell suspension) has to be

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Abbreviations: DSC - differential scanning calorimetry; LN - liquid nitrogen; LS - loading solution, MS - Murashige and Skoog; PVS - plant vitrification solution; SSR - simple sequence repeats.

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protected by dehydration before freezing in contrast to the direct cryopreservation of some plant material in which natural dehydration processes occur (Benelli *et al.* 2013). Vitrification-based techniques represent a simplified cryogenic procedure based on plant tissue dehydration achieved by treatment with cryoprotectant compounds following by immersion in highly concentrated vitrification solutions prior to storage in LN (Danso and Ford-Lloyd 2011). In addition, the vitrification enables the transition of water from the liquid phase into an amorphous phase or glassy-state structure to avoid the nucleation of the ice crystals (Fahy *et al.* 1984). The phase transitions of the plant materials associated with temperature change and heat flow are measured by differential scanning calorimetry (DSC), which precisely monitors the heat quantity radiated or excessively absorbed by the sample during a change of temperature within a time (Gill *et al.* 2010). To date, a thermal analysis approach in combination with the vitrification technique has been applied throughout the plant kingdom, also including woody plant species (Ryynänen and Aronen 2005, Vidal *et al.* 2005, Uchendu *et al.* 2010, Gantait *et al.* 2015).

A widely used plant vitrification solution 2 (PVS2) containing 30 % (m/v) glycerol, 15 % (m/v) polyethylene glycol, and 15 % (v/v) dimethylsulfoxide in a liquid Murashige and Skoog (MS, 1962) medium with 0.4 M sucrose (Sakai *et al.* 1990) was also applied during the vitrification procedures of *Populus* spp. germplasm preservation. Jokipii *et al.* (2004) tested three different cryopreservation protocols and found that the slow cooling method used for *P. tremula* × *P. tremuloides* buds *in vivo* resulted in the highest regeneration percentages (72 - 96 %) in comparison to PVS2 treatment (22.5 - 65 %), whereas slow cooling in polyethylene glycol, glucose and dimethylsulfoxide solution containing 10 % (m/v) polyethylene glycol 6000, 10 % (m/v) glucose, and 10 % (v/v) dimethylsulfoxide used for *in vitro* grown apical segments resulted in a recovery percentage of 1.1 %. Alternatively, Lambardi *et al.* (2000) presented an efficient one-step vitrification procedure using PVS2 for the shoot tips of *P. alba* with almost 60 % of the rooted shoots originating from LN storage. A similarly high post-freeze survival rate (78 %) of white poplar shoot tips after using the PVS2 vitrification protocol was demonstrated by Tsvetkov *et al.* (2009). Interestingly, several studies reported a superior effect of alternative plant vitrification solutions modified from the original PVS2 by increasing the glycerol and sucrose concentrations and/or omitting DMSO and polyethylene glycol in many plant species (Storme *et al.* 2004, Kim *et al.* 2009, Kaviani 2011, Nuc *et al.* 2016), but currently, no data on the PVS3 application are available for poplar species.

Among the factors that may substantially affect the effectiveness of the germplasm cryopreservation are appropriate plant genotypes, which are currently generally characterized by molecular markers (Sales *et al.* 2001). The selection of valuable genetic resources for cryopreservation and the determination of genetic diversity at a group or subgroup using the simple sequence repeats (SSR) method was widely applied to, *e.g.*, dessert

apple germplasm (Urrestarazu *et al.* 2016) or avocado species (Guzmán *et al.* 2017). In *Populus*, the patterns of genetic variation described by the SSR markers have been developed to estimate the genetic structure within and among populations, species, and hybrids, or for other purposes (Storme *et al.* 2004, Van Loo *et al.* 2008, Liesebach *et al.* 2010, Politov *et al.* 2015) but not in terms of the long-term storage of poplar germplasm.

The objective of this study was to develop a cryopreservation method appropriate for the conservation of a unique grey poplar genotype based on a vitrification procedure and previous explant acclimation. The SSR method was used to identify genotypes within the grey poplar population, and differential scanning calorimetry was used to determine the optimal tissue hydration. The hypothesis that explant preculture, increasing their tolerance to dehydration, and a significant reduction in the freezable water content can improve the cryotolerance of grey poplar shoot tips has been tested.

Materials and methods

Genotype selection using nuclear microsatellite markers: Thirty samples of grey poplar (*Populus* × *canescens* Aiton Sm.) individuals were collected from the floodplain forest at the Dyjávovice village in the South Moravia, Czech Republic. Total genomic DNA was extracted from 20 mg of dry young leaf using a DNeasy plant mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Quality and quantity of DNA was determined using a spectrophotometer (NanoPhotometer, Implen, München, Germany) and stored at -20 °C. Genetic variation was analysed by using the nine previously characterized polymorphic nuclear microsatellite markers ORPM16, ORPM220, ORPM30, ORPM312, WPMS15, WPMS5, ORPM60, WPMS16, and ORPM20 (Van der Schoot *et al.* 2000, Smulders *et al.* 2001, Tuskan *et al.* 2004, Van Loo *et al.* 2007; http://www.ornl.gov/sci/ipgc/ssr_resource.htm). Fluorescently labelled primers have been sorted into the two multiplexes and used for fragment amplification. Polymerase chain reaction cycles consisted of a denaturing step at 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 45 s, with a final elongation step at 72 °C for 20 min. The size of the amplified fragments was determined on an Applied Biosystems (Foster City, CA, USA) 3500 sequencer using the capillary electrophoresis method. The alleles were sized based on the GeneScan™ – 600 LIZ® size standard v. 2.0 (Applied Biosystems), subsequently evaluated by the GeneMapper software (Applied Biosystems), and individually verified. Multilocus match analyses were evaluated by GenAlEx v. 6.502 (Peakall and Smouse 2012). The allele frequency analysis, including the number of total alleles scored (Na), observed heterozygosity (Ho), expected heterozygosity (He), polymorphic information index (PIC) and the null allele frequency (F null), were estimated by the program Cervus 3.0.7 (Kalinowski *et al.* 2007). The most abundant genotype occurring among the individuals of the grey

poplar population was defined as the representative plant material for the following cryopreservation experiments.

Explant establishment and culture: Grey poplar (*Populus ×canescens* Aiton Sm.) twigs bearing dormant buds (clone 18) were collected at the beginning of March from one tree more than 40 years old at Dyjákovice Village (N 48,78442°, E 16,29252°) in South Moravia, Czech Republic. After transfer into the laboratory, sterilization procedures of the dormant buds were followed in several steps: incubation for 20 min in *Tween*®20 (2 droplets per 10 cm³), 20 min in disinfecting solution *KORSOLEX*®plus (25 cm³ per 500 cm³ of distilled water), rinsing for 20 min in running tap water, 20 min incubation in HgCl₂ (1 mg dm⁻³), and subsequent removal to a laminar flow hood where they were submerged in sterile water three-times for 15 min. To induce organogenesis, isolated shoot tips from dormant buds were placed on a modified MS medium enriched with 10 mg dm⁻³ glutamine, 2 mg dm⁻³ glycine, 0.2 mg dm⁻³ 6-benzylaminopurine (BAP), and 0.1 mg dm⁻³ indole-3-butyric acid (IBA), containing 30 g dm⁻³ sucrose and 6 g dm⁻³ agar, with the pH adjusted to 5.8 with 1 M KOH. The segments were cultivated in controlled conditions at 24 °C, a 16-h photoperiod, and an irradiance of 30 µmol m⁻² s⁻¹. After approximately four weeks, the proliferation of the shoots was determined. Subculturing and multiplication of the explants was performed every third week on the same medium to ensure sufficient amounts of vital plant material.

Explant acclimation and vitrification procedure: *In vitro* cultures of grey poplar were divided into three groups (treatments A, B, C) differing in preculture conditions, including different culture temperatures and osmotic treatments. Treatment A represented *in vitro* grown shoots, which were maintained in control conditions (24 °C, 16-h photoperiod, and an irradiance of 30 µmol m⁻² s⁻¹). The shoots of treatments B and C were cold hardened at 6 °C for four weeks under the same other conditions. The treatment C involved addition of 0.7 M sucrose (12.5 cm³ was added as a solution on the top of 25 cm³ of agar medium into each glass jar by pipetting) for the third and fourth weeks of cold hardening, thus the final sucrose concentration, including sucrose in both, medium and solution, reached 0.3 M in each culture vessel.

After four weeks of precultivation, isolated apical shoot tips (approximately 200 pieces of 2 - 3 mm length per treatment) were transferred into the Petri dishes (10 plant segments in one Petri dish) containing modified agar MS medium enriched with 0.3 M sucrose and placed into a cultivation room overnight (24 °C). A modified vitrification protocol described by Nishizawa *et al.* (1993) was applied with some modifications. Isolated shoot tips were transferred into 10 cm³ of loading solutions; LS [2 M glycerol and 0.4 M sucrose, pH 5.8] for 30 min at room temperature. The LS was removed and replaced with 10 cm³ of a half-concentrated plant vitrification solution 3 (PVS3; 2.7 M glycerol and 0.073 M sucrose) for 30 min. Subsequently, the PVS3 solution was removed and replaced with 10 cm³ of modified PVS3 vitrification solution (80 %

PVS3 containing 4.34 M glycerol and 1.17 M sucrose). The samples were dehydrated by either 120, 150, or 240 min, but they were not treated with liquid nitrogen. Before their recovery on growth medium, the shoot tips were treated with unloading solution containing 0.75 M sucrose for 30 min to avoid their osmotic damage. Finally, ten shoot apices from each treatment were transferred to Petri dishes containing a modified MS medium enriched with phytohormones and other substances as described above for the induction and multiplication of the explants and placed into the growth room under the same conditions as described above but covered by a sheet of paper to prevent photoinhibition. Two weeks later, the survival of the shoot apices was evaluated. Segments were transferred into the same fresh medium where they remained until eight weeks for the final evaluation of the shoot tips regrowth percentage.

Ultra-rapid cooling by using liquid nitrogen: Isolated apical segments, pretreated accordingly as mentioned for treatment C and dehydrated in PVS3 for 180 or 240 min, were directly transferred onto aluminium rectangles (20 × 6 × 0.05 mm) and cryopreserved by ultra-rapid cooling with liquid nitrogen LN (-196 °C). The highly viscous PVS solution tightly stuck the shoot tips to the aluminium rectangle without risk of their loss in the LN. They were in LN for a minimum of 1 h. The individual rectangles with 10 shoot tips were quickly plunged into the 2 cm³ of polypropylene cryovials (*Nunc, Fisher Scientific, location?*) filled with LN for 60 min. Samples were quickly thawed by transferring the aluminium foils from LN into a heated (40 °C) unloading solution of the same composition as described above. Ten shoot tips were subsequently transferred onto Petri dishes (5 cm diameter) containing a modified MS medium enriched with phytohormones and other substances as described above, moved into the growth chamber, and covered with a sheet of paper to prevent photoinhibition. The shoot tip survival and regrowth were evaluated after two and eight weeks, respectively.

Measurement using DSC: Thermal analysis by the differential scanning calorimetry was performed during dehydration with cryoprotective solutions under different preculture conditions. The proportion of freezable water (percent of the fresh mass) was determined using a differential scanning calorimeter *Q2000* with a refrigerated cooling system (*TA Instruments*, New Castle, DE, USA) in the temperature range from -90 to +20 °C at cooling and heating rates of 10 °C per min with the use of hermetically sealed aluminium pans.

Data collection and statistical analysis: Three independent biological repetitions were conducted for all treatments with at least 10 samples per replicate. The data collected were evaluated using one-way analysis of variance. Statistically significant differences among the individual treatments are recorded based on all pairwise multiple comparison procedures according to the Holm-Sidak method at $\alpha = 0.05$ (*SigmaPlot 14.0, Systat Software*,

Hounslow, UK).

Results

Patterns of genetic diversity variation in thirty phenotypically valuable grey poplars growing at the floodplain forest in the South Moravia region of the Czech Republic were evaluated to select the most frequently occurring genotype among the individuals for further cryopreservation procedures. The nine SSR markers used in this study detected a total of 37 alleles ranging from 2 to 7 alleles, with an average number of total alleles scored representing 4.11 per locus. The most polymorphic loci exhibit ORPM30 and WPMS15 with polymorphic information index 0.527 and 0.524, respectively, in contrast to the ORPM16 and ORPM312 loci (Table 1). According to the genetic characteristics evaluated, a very low genetic variability was detected in the collection of samples in this study (Table 1). In addition, the multilocus matches of the microsatellite analysis revealed only 5 different genotypes in the group of 30 individuals of selected grey poplars. Individuals of the female sex, which were determined later during flowering time, represented 28 samples with the most prevailing genotype detected for 26 of them (online resources 1). In summary, our data point to a low allelic diversity in the population studied and reveal a clonal identity for 26 grey poplars, which we consequently used as a representative clone for our long-term storage experimental study.

Apical shoot tips isolated from non-acclimated explants of grey poplar (treatment A) tolerated their exposure to 0.3 M sucrose in modified MS medium for overnight and subsequent LS or PVS3 treatments for 30 min without significant decrease in their regrowth and regeneration. However, they showed a decline in their regrowth and regeneration after PVS3 treatment for 120, 180, and 240 min when compared to treatments B and C. The minimal average two-week recovery of shoot tips from non-acclimated explants dropped to 23.3 ± 5.8 % (Fig. 1A). In cold-acclimated explants (treatment B), tolerance to dehydration of isolated shoot tips improved significantly, when compared to the non-acclimated explants, and the minimal average explant regrowth, after two-week recovery, increased to 65 ± 21.2 % (Fig. 1A). Simultaneous effect of cold acclimation and osmotic treatment with sucrose solution (treatment C) had the highest positive effect on increase of dehydration tolerance which increased to 83.3 ± 15.3 % (Fig. 1A). The increase in dehydration tolerance of isolated grey poplar shoot tips after explant acclimation by simultaneous cold and osmotic treatment was confirmed after eight-week recovery (Fig. 1B). These explants exposed to PVS3 for 120 and 150 min achieved the highest regrowth percentage (93.3 and 90.0 %, respectively) in comparison to non-acclimated (60 ± 10 % and 46.7 ± 5.8 %, respectively) and cold-acclimated explants (56.7 ± 20.8 % and 56.7 ± 15.3 %) (Fig. 1B). However, the shoot tip regrowth substantially decreased after 240 min of PVS3 exposures in treatment C up to 83.3 ± 15.3 %. Our results confirmed the positive effect of

cold and osmotic acclimation on dehydration tolerance of isolated grey poplar shoot tips.

The freezable water content in isolated shoot tips was influenced by pre-culture conditions (treatments A, B, and C). Non-acclimated control samples (treatment A) had a higher water content (85.3 ± 9.1 %) than the acclimated plants (treatments B and C) containing 73.2 ± 4.5 % and 64.5 ± 2.3 % of freezable water, respectively. Subsequent 30 min treatments with LS and PVS3 caused a decline of the freezable water content of isolated shoot tips in all treatments and the sharpest reduction was observed after the 120 min PVS3 treatment (Fig. 2). Following incubation in PVS3 slowly dehydrated the plant samples across the 120 - 240 min interval (Fig. 2), but these changes in freezable water content were not statistically significant. The content of freezable water after the PVS3 solution exposure decreased from 5.6 ± 0.4 to 1.7 ± 1.6 % in treatment A, whereas from 4.1 ± 1.3 to 0.8 ± 0.3 % and from 4.3 ± 1.3 to 0.9 ± 0.1 % in treatments B and C, respectively. Our results demonstrate that pre-cultivation conditions and the subsequent cryoprotectant treatment had significant effects on the content of freezable water in plant tissues. It was proved that PVS3 was very efficient in dehydration of isolated shoot tips of grey poplar.

Two important findings, 1) simultaneous cold and osmotic acclimation increased dehydration tolerance (treatment C) and 2) successful PVS3 dehydration reduced freezable water content, were employed to test their effect on grey poplar cryotolerance. Due to a lack of significant differences among the regeneration rate of the explants after dehydration for 120 min (93.3 ± 5.8 %) and 240 min (83.3 ± 8.8 %) in PVS3 (Fig. 3), a shorter interval between 180 min and 240 min (in the end of PVS3 dehydration period) was selected to reach the optimal dehydration for the maximal post-thaw recovery. Prolonged exposure of PVS3 treatment allowed to reduce the freezable water content (approximately 1 %), which has not resulted in explant damage by excessive dehydration yet (Fig. 3). Cryopreserved apical shoot tips incubated for 180 min and 240 min in the cryoprotective solution exhibited only a slight difference on the regrowth of the shoot tips after two-week recovery (Fig. 4). After eight-week recovery, post-thaw regeneration reached 83.3 ± 15.3 % and 90 ± 0 % in explants incubated in PVS3 for 180 and 240 min, respectively (Fig. 3). The statistically significant difference in explants regeneration after 180 and 240 min of cryoprotective solution treatment was not proved. In summary, even though no significant differences have been observed among the shoot tips treated by PVS3 cryoprotectant for 180 or 240 min, the regrowth percentage of grey poplar after thawing from the liquid nitrogen was higher than 80 % after 8 weeks of evaluation.

Discussion

The aim of this work was a preservation of valuable genotypic and phenotypic traits of grey poplar (*Populus × canescens* Aiton Sm.) originating from a unique population located in a floodplain forest in the South Moravia region

Table 1. Genetic diversity parameters in selected 30 grey poplar (*Populus ×canescens* Aiton Sm.) individuals. Na - number of observed alleles, Ho - observed heterozygosity, He - expected heterozygosity, PIC - polymorphic information content, HW - Hardy-Weinberg expectations, F null - null allele frequency, ND - not determined, **, *** deviations from HW significant at 0.01 and 0.001 probability level, respectively.

Locus name	Na	Ho	He	PIC	HW	F null
ORPM220	3	1	0.54	0.421	***	-0.3154
ORPM16	3	0.1	0.098	0.094	ND	-0.0161
WPMS5	5	0.067	0.247	0.235	ND	0.6264
WPMS15	5	1	0.615	0.524	***	-0.2656
ORPM20	5	0.967	0.572	0.466	***	-0.2894
WPMS16	5	1	0.601	0.506	***	-0.2747
ORPM30	7	1	0.616	0.527	***	-0.2652
ORPM312	2	0	0.127	0.117	ND	0.8138
ORPM60	2	0.867	0.499	0.371	**	-0.2765

Fig. 1. The effect of pretreatment conditions on the regrowth of the grey poplar (*Populus ×canescens* Aiton Sm.) shoot tips evaluated after two (A) and eight (B) weeks of cultivation on a modified Murashige and Skoog medium. Pretreatment conditions: treatment A - no acclimation, treatment B - cold hardening (6 °C), treatment C - cold hardening (6 °C) with incubation in sucrose (0.3 M). LS - loading solution, PVS3 - modified plant vitrification solution. Means ± SEs, $n = 3$, different letters indicate statistically significant differences at $P < 0.05$.

of the Czech Republic and endangered by attacks of fungal pathogens. The first task, the determination of the genetic diversity of grey poplar, was performed using nine polymorphic microsatellite loci (SSR markers), which discriminated the repeated genotypes among the 30 selected individuals (Table 1 Suppl.). According to the reduced area of sampling (the longest distance between samples was 2.5 km) the number of individuals analysed (30) and the number and type of SSR markers used (9 SSR), we found a clonality prevalence for 26 of them, the high frequency (more than 86 %) of the most abundant genotype in the population showed that this population was more clonal than sexual. Low allelic diversity was observed for an additional 125 samples collected in the same area where a clonal identity for most of the samples was determined using 5 additional SSR markers (Pokorná *et al.* 2018). Similarly, Fussi *et al.* (2012) found identical genotypes of *Populus alba* in all 28 samples taken from different valleys of the island Malta, one of the grey poplar parents. In addition, it is generally known that the genus *Populus* is characterized by a high ability for clonal propagation primarily by sprouting, which leads to a broad extension of only one clone on the area as reported, *e.g.*, Kemperman and Barnes (1976) and DeWoody *et al.* (2008), for the giant clone of *Populus tremuloides* occupying 4 330 m² and containing 47 000 ramets. Interestingly, we identified a disproportion in the female and male sex occurrence during the collection of samples with the most abundant female sex characterized for 26 of them by the same allele sizes determined by the 9 SSR markers. Currently, the detection of diversity in monoclonal poplar stands is linked to the application of other approaches than using the SSR method, such as epigenetic diversity determining the DNA methylation status (Guarino *et al.* 2015) or looking for single nucleotide polymorphism markers (SNPs), which

appear to exhibit a slightly higher average of differentiation than the other types of codominant markers (Ingvarsson *et al.* 2005, Keller *et al.* 2010, Olson *et al.* 2010).

The predominant and representative genotypes (Table 1) of the native grey poplar population was selected for their preservation due to its valuable features such as a narrow crown and a straight continuous trunk with natural pruning (Buriánek and Novotný 2016). The high frequency of this genotype in the population indicates its very high vitality and competitiveness in spreading in the environment. Thus, in addition to its valuable properties, this genotype has also a very high potential for its successful cultivation in forests. It has been shown, using five chestnut clones, that genotype specificity might substantially affect shoot recovery after thawing from the liquid nitrogen temperature from 38 - 54 % (Vidal *et al.* 2005), which has also been reported for 6 lines of *in vivo* hybrid aspen buds, in which the regrowth rate ranged from 59 - 95 % and 72 - 96 % after 2 and 4 weeks of cultivation, respectively (Jokipii *et al.* 2004). Similarly, a variable regeneration rate of shoot tips was observed among four apple cultivars non-cryopreserved as well as after storage in liquid nitrogen (Halmagyi *et al.* 2010).

In our study, we used a vitrification procedure employing the modified PVS3 solution for the first time to test its effect on the post-thaw recovery of grey poplar shoot tips. The success of cryopreservation is influenced by the sensitivity of the genotype to a particular cryopreservation process, and usually involves sensitivity to strong dehydration of the tissues (Benson 2008). Because the cryotolerance is not a natural ability of any species, improvement of dehydration tolerance can be a way how to cope with the cryopreservation conditions. An assumption that a high dehydration tolerance allows a very massive water content decrease which limits ice development and growth during

Fig. 2. The effect of pretreatment conditions on the content of freezable water content in grey poplar (*Populus × canescens* Aiton Sm.) shoot tips. Pretreatment conditions: treatment A - no acclimation, treatment B - cold hardening (6 °C), treatment C - cold hardening (6 °C) with incubation in sucrose (0.3 M). LS - loading solution, PVS3 - modified plant vitrification solution. Means ± SEs, *n* = 3, different letters indicate statistically significant differences at *P* < 0.05.

explants cooling to an ultra-low temperature (-196°C) can be defined. Thus, we formulated the hypothesis that grey poplar explant acclimation by cold or osmotic treatments and following decrease in freezable water content improve their cryotolerance.

Three different preculture conditions were tested: A - no acclimation, B - cold hardening, and C - simultaneous

effect of cold hardening and osmotic treatment. Treatments employing a low temperature acclimation (Bilavcik *et al.* 2012) or a simultaneous low temperature and osmotic treatment (Faltus *et al.* 2011) have been used successfully for acclimation of explants before their cryopreservation. In our study, treatment A had no significant effect on the explant regrowth, but it resulted in the different sensitivity

Fig. 3. The effect of plant vitrification solution 3 application on grey poplar (*Populus × canescens* Aiton Sm.) shoot tips after dehydration and then cryopreservation in liquid nitrogen. The shoot tips were pretreated by cold hardening with the application of 0.7 M sucrose. The regrowth and content of freezable water was evaluated after eight weeks of segment cultivation on a modified Murashige and Skoog medium. Means $\pm$ SEs, $n = 3$. No significant differences in the regrowth percentage was found.

Fig. 4. The effect of plant vitrification solution 3 (PVS3) application on grey poplar (*Populus × canescens* Aiton Sm.) shoot tip regrowth after cryopreservation in liquid nitrogen evaluated after two weeks of cultivation on a modified Murashige and Skoog medium. The shoot tips were pretreated by cold hardening (6°C) and 0.3 M sucrose followed by incubation for 180 min (A - C) and 240 min (D - F) in 80 % PVS3. The diameter of Petri dish was 70 mm.

of explants to dehydration using the cryoprotective solution PVS3 (Fig. 1). However, the explant acclimation significantly decreased the freezable water content determined by DSC. Similarly, a frozen water content decrease was measured by DSC in encapsulated *Theobroma cacao* somatic embryos subjected to 0.08 - 1.25 M sucrose (Fang *et al.* 2009). The decrease in freezable water content confirmed the ability of grey poplar explants to acclimate to a low temperature and osmotic stress. This reduction in the freezable water content can occur due to a decrease in the total water content and/or osmotic adjustment, *i.e.*, the accumulation of osmotically active substances such as soluble sugars (Beck *et al.* 2007).

The next steps of the vitrification procedure including the treatment of isolated shoot tips with 0.3 M sucrose overnight, 30 min treatment with LS and following 30 min treatment with PVS3 did not significantly decrease the regrowth of the explants, but the last treatment resulted in a significant decrease in freezable water content (Fig. 1). Gradual tissue dehydration as well as employment of 80 % PVS3 instead of concentrated PVS3 solution helps avoiding the strong osmotic stress and explants injury during vitrification protocol (Faltus *et al.* 2015). Any significant differences in the freezable water content and melting temperature among the three experimental treatments tested were not detected in the individual steps of the vitrification procedure with the exception of the preculture treatments described above. That shows the decisive effect of particular cryoprotective solutions on thermal characteristics at steady-state conditions after 30 min of their treatments.

The final step of the vitrification procedure, a strong dehydration by the cryoprotective solution PVS3, significantly decreased the freezable water content in all the treatments tested. The decrease in the water content is a prerequisite for successful cryopreservation, but it can result in explant injury due to dehydration or the cryoprotectant influence (Valk and Walters 2006). Grey poplar explant dehydration resulted in a decrease in their regrowth, but the reduction depended on the preculture conditions tested. Maximal recovery of explant treated with PVS3 and evaluated after 2 weeks of shoot tip regrowth reached 30.0 ± 0.0 %, 76 ± 25.2 %, and 95 ± 7.1 % in treatments A, B, and C, respectively (Fig. 1A). The most sensitive to dehydration were the non-acclimated explants, which exhibited the statistical significant difference compared to treatments B and C after both two and eight weeks of regrowth (Fig. 1B). The explants from treatment C exhibited the highest regrowth percentage (93.3 ± 5.8 % after 120 min of PVS3 exposure) after 8 weeks compared to treatments A and B, which is higher than after the PVS2 application at 0 °C for 60 min resulting in a survival percentage of 90 % of the white poplar axillary buds precultured on the MS medium containing 0.09 M sucrose (Caccavale *et al.* 1998). However, not only temperature and the time of plant vitrification solution exposures are important factors for plant regeneration, as reported by Lambardi *et al.* (2000), who showed that sucrose concentration in the preculture medium could also significantly reflect the shoot tip survival of white poplar.

Thus, grey poplar is a very suitable woody plant species to follow the individual cryoconservation procedures in a short time due to a fast shoot proliferation and multiplication of the explants as well as the low requirements of the specific cultivation conditions such as a composition of nutrients or phytohormones in a medium (Žižková *et al.* 2017). Similarly, we observed quickly developing green plantlets on the modified MS medium two weeks after thawing (Figs 3 and 4). Treatment C was selected for the experiments with liquid nitrogen. The explant recovery after the LN treatment was 76.7 ± 5.8 % and 73.3 ± 20.8 % after 2 weeks for explants dehydrated for 180 and 240 min, respectively, and after 8 weeks, it was 83.3 ± 15.3 % and 90 ± 0.0 %, and the differences between the two exposure times of PVS3 treatment were not significant in both terms of explant evaluation. A rapid immersion in LN and rewarming samples maintained a high regeneration of grey poplar explants treated for 240 min in PVS3 up to 90 %, which is comparable with the regeneration of the non-cryopreserved shoot tips from treatment C varying from 93.3 ± 3.3 to 83.3 ± 8.8 % (Fig. 3). This finding shows that the regeneration potential of the dehydrated explants was maximally utilized and no serious injury of the poplar explants caused by liquid nitrogen was observed. We can summarize that an appropriate way of explant acclimation and an efficient decrease in freezable water content can shift the biological limits of cryotolerance in the plant kingdom.

Conclusions

Cryogenic techniques and molecular genetics were combined to preserve a representative clone of grey poplar population (*Populus × canescens* Aiton Sm.) valuable for its phenotype traits and as a source for the replacement of withering and dying ash and alder stands attacked by fungal pathogens at several localities of the floodplain forest in the Czech Republic. The hypothesis that a specific explant preculture and a decrease in the freezable water content improve the cryotolerance of grey poplar shoot tips has been proved. Simultaneous cold and osmotic treatments increased grey poplar dehydration tolerance, which allowed a considerable freezable water content decrease without a serious injury of explants. For the first time, a vitrification procedure based on the PVS3 cryoprotective solution was successfully tested for the cryopreservation of grey poplar explants. The results achieved will allow saving the unique, the most abundant genotype of grey poplar in the South Moravia region, and they will help to recover and expand this genotype in the future.

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