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MICROPROPAGATION

First successful vitrification-based cryopreservation of *in vitro* shoot tips of Sycamore Maple (*Acer pseudoplatanus* L.): Influence of explant size, hardening, and pre-culture

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Abstract

This study presents the first reproducible vitrification-based cryopreservation protocol for shoot tips of sycamore maple (*Acer pseudoplatanus* L.), a tree of high ecological and economic significance in Central Europe. By safeguarding genetically verified material, including rare wood traits, this work supports the integration of the species into long-term *ex situ* conservation programmes. We investigated the influence of explant size, hardening duration, daytime temperature during hardening, and pre-culture across four genotypes. The protocol involved 4 wk of cold hardening under short-day conditions, a pre-culture at low temperature, vitrification with a plant vitrification solution, storage in liquid nitrogen, rapid rewarming, and gradual recovery under controlled light. Explant size proved decisive: shoot tips of 1–2 mm in length regrew at substantially higher rates and developed more vigorous shoots than larger explants. Four weeks of hardening with daytime temperatures between 3 and 7°C followed by pre-culture at 5°C produced the most reliable outcomes. The most responsive genotype reached regrowth in up to 90% of the explants, and for the remaining three genotypes, 50–64% regrowth was achieved. Post-cryopreservation, multiplication factors were comparable to non-cryopreserved control. Hyperhydricity occurred in a genotype-dependent manner but remained low overall. Thus, the protocol enables cryopreservation of sycamore maple genotypes, ensuring both survival and propagation potential.

Keywords *Acer pseudoplatanus* · Ex situ conservation · Gene bank · Liquid nitrogen · Regrowth

Introduction

The conservation of plant genetic resources is a global priority in the face of accelerating biodiversity loss, climate change, and increasing demands on agricultural and forest systems. Forest trees play a pivotal role in ecosystem resilience and the provision of important ecosystem services, yet their long life cycles and complex genetic structures pose significant challenges for long-term conservation (Lefèvre *et al*. 2013; Commission on Genetic Resources for Food and Agriculture, FAO 2014). *Ex situ* strategies, such as gene banks, are essential for safeguarding the genetic diversity of economically and ecologically important species. While seed storage is widely applied, it is often not feasible for tree species with recalcitrant seeds or for clonally propagated genotypes (Berjak and Pammenter 2007; Engelmann 2011). For such cases, cryopreservation, defined as ultra-low-temperature storage in liquid nitrogen (LN), is increasingly recognized as a complementary tool within integrated conservation frameworks (EUFORGEN 2021).

Cryopreservation has been successfully applied to a wide range of plant materials, including seeds, pollen, dormant buds, embryogenic cultures, and shoot tips (Engelmann and Dussert 2013; Engels and Ebert 2021). It provides genetically stable, pathogen-free storage for maintained *in vitro* plant material over decades without the need for subculturing (Niino *et al*. 1992; Engelmann 2011). Compared to agricultural and horticultural crops, information on factors driving success in cryopreservation is scarce for woody species, especially forest trees (Li *et al*. 2018). Protocols have been reported for some temperate hardwoods, but the

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number of species with established cryopreservation procedures remains small, and recovery rates vary considerably among taxa and genotypes (Corredoira *et al*. 2017; Li *et al*. 2018; Cherian and Thomas 2024).

Among temperate broadleaved species, sycamore maple (*Acer pseudoplatanus* L.) is of particular interest. As a keystone broadleaved species in Central European forests, it contributes to biodiversity, structural diversity, and soil protection (Kiebacher *et al*. 2017). Its adaptability and high intra-population genetic diversity (Pandey *et al*. 2012) support its role in climate-resilient forestry and justify its strategic inclusion in *ex situ* forest genetic resource conservation programmes. Beyond its ecological value, sycamore maple yields high-quality timber, with wavy-grain phenotypes commanding exceptional market prices (Conrad 1957, 1977; Beals and Davis 1977). However, conventional *ex situ* conservation methods are constrained by seed recalcitrance, pests, and environmental risks. This makes cryopreservation of vegetative tissues a promising complementary strategy for safeguarding genetically verified material, including rare and high-value traits such as wavy grain.

Progress has been made in the cryopreservation of shoot tips of fruit tree species, including *Malus* and *Pyrus* (Niino *et al*. 1992; Li *et al*. 2015) as well as *Prunus* (Niino *et al*. 1997). Similar advances have been reported for forest tree species, such as *Fraxinus excelsior* (Schoenweiss *et al*. 2005) and *Betula* spp. (Meier-Dinkel 2007). In contrast, protocols for species in the genus *Acer* remain scarce. The work focused on embryonic axes, with sycamore maple showing high dehydration sensitivity relative to other *Acer* species (Pukacki and Juszczyk 2014). Because approaches based on seeds or zygotic embryos cannot ensure clonal identity, *in vitro* shoot tips are preferred for preserving elite genotypes. Currently, no reproducible method exists for cryopreservation of *in vitro* shoot tips of sycamore maple, limiting its secure integration into forest genetic resource collections. Thus, developing an efficient cryopreservation protocol would provide a valuable option for preserving genetically important material of this species.

A range of approaches has been developed for the cryopreservation of plant tissues. In slow-cooling systems, temperature is gradually lowered following cryoprotectant application, allowing extracellular ice formation while minimizing intracellular damage (Panis and Lambardi 2006; Engelmann 2011). Encapsulation–dehydration embeds explants in alginate beads, followed by osmotic and physical dehydration before rapid freezing (Fabre and Dereuddre 1990). Vitrification, in contrast, achieves a glassy, ice-free state upon ultra-rapid cooling after dehydration in highly concentrated plant vitrification solutions (PVS), such as PVS2, which contains glycerol, ethylene glycol, dimethyl sulfoxide, and sucrose (Sakai *et al*. 1990). This method is particularly effective for small, organized tissues such as shoot tips and is widely used for woody species due to its high survival rates and broad applicability (Towill 1995; Panis 2019).

Physiological pre-treatments such as cold hardening and osmotic pre-culture can enhance the tolerance of plant tissues to dehydration and ultra-low temperatures. Cold hardening under controlled photoperiod and temperature regimes induces metabolic and structural adaptations, including the accumulation of soluble sugars, changes in membrane composition, and increased cytoplasmic viscosity, all of which reduce the likelihood of damaging ice formation (Benson 2008). In some temperate woody species, these effects can be further strengthened by the application of abscisic acid (ABA) during hardening, which has been shown to increase recovery rates after cryopreservation, although responses were genotype-dependent (Ryynänen 1998). Osmotic preculture in cryoprotectant-rich media facilitates controlled water loss and osmotic adjustment, thereby reducing intracellular ice nucleation during cooling (Benson 2008). Explant size and genotype also influence post-thawing survival and regeneration, but their specific roles in sycamore maple remain unknown.

The aim of this study was therefore to develop and optimize a vitrification-based cryopreservation protocol for *in vitro* shoot tips of sycamore maple by systematically assessing the effects of hardening duration, day temperature during hardening, pre-culture duration, and explant size across multiple sycamore maple genotypes. The findings are intended to support the integration of this species into long-term *ex situ* conservation programmes for temperate woody germplasm.

Materials and methods

Plant material and general culture conditions Unless specified otherwise, all experiments were conducted with 4-wk-old *in vitro* shoots of four sycamore maple genotypes. These genotypes originated from donor trees with distinct wood characteristics: (i) E87 was derived from seedling material (1–2 yr old) and introduced into *in vitro* culture in 2016 by the Thünen Institute of Forest Genetics, Waldsieversdorf, Germany. It most likely represents straight-grained wood. (ii) Gött47-38 was established *in vitro* in 2012 from scion wood of a tree grafted in 1992 with seed orchard material from the Göttingen municipal forest, Germany. (iii) Schussi originated from crown material collected in Vorarlberg (Austria) and was established *in vitro* in 2016 by the Thünen Institute of Forest Genetics, Waldsieversdorf, Germany. (iv) SFHb1 was derived from stump sprouts collected in the Heilbronn municipal forest, Germany and was transferred into *in vitro* culture in 2017. As the donor trees of Gött47-38,

Schussi, and SFHb1 displayed wavy-grained wood, these three genotypes are regarded as potential wavy-grain candidates. Donor plants were maintained by regular subculturing of shoot tips and nodal segments every 5 wk onto a propagation medium consisting of half-strength Murashige and Skoog (MS) salts (Murashige and Skoog 1962) combined with half-strength Rugini Olive salts (Rugini 1984) (both Duchefa Biochemie, Haarlem, The Netherlands), supplemented with 2% sucrose, 11.8 µM silver nitrate, 0.025 µM indole-3-butyric acid (IBA), and 13.69 µM trans-zeatin. Trans-zeatin was sterilized by filtration through a 0.22-µm membrane filter and added to the medium after autoclaving (121°C, 15 min), once cooled to approximately 50°C. The medium was solidified with 0.6% Phyto agar (Duchefa Biochemie) and adjusted to pH 5.7 prior to autoclaving. Cultures were grown in 370-mL glass jars (Sturz-Glas, J. WECK GmbH & Co. KG, Wehr-Öflingen, Germany), each containing approximately 70 mL of medium, with ten explants per vessel. A nonwoven fabric ring was placed between the jar and the glass lid to allow gas exchange. Cultures were incubated at 22 °C under a 16-h photoperiod, with a light intensity of ~ 20 µmol m^{-2} s^{-1} provided by LED lamps (spectrum: AP67, Valoya, Helsinki, Finland).

General steps of the cryopreservation protocol – Hardening The beginning of the hardening period was defined as the start of each experiment. For that, 4-wk-old *in vitro* shoots were subjected to hardening treatments in a growth chamber under short-day conditions (8-h photoperiod), with a light intensity of approximately 15 µmol m^{-2} s^{-1} provided by the LED lamps specified before. Day and night temperatures were adjusted according to the specific experimental treatments (see below).

Pre-culture Unless stated otherwise, shoot tips of 1 to 2 mm length were excised from *in vitro* shoots and placed on solid pre-culture medium (30 mL per dish) in polystyrene Petri dishes (94 × 16 mm) with ventilation lugs (Greiner Bio-One, Frickenhausen, Germany; ten explants per Petri dish). The pre-culture medium consisted of half-strength MS salts (Murashige and Skoog 1962) and half-strength Rugini Olive salts (Rugini 1984) (both Duchefa Biochemie), 3% sucrose, and 7 g L^{-1} Phyto agar (Duchefa Biochemie). All components were used for 1 L, but the solution was adjusted to 800 mL, the pH was set to 5.7, and then, it was autoclaved. A 4 M glycerol stock solution (73.7 g glycerol dissolved in deionized water and brought to 200 mL), sterile-filtered, was added aseptically to the 800 mL basal medium, resulting in 1 L of final medium supplemented with 0.8 M glycerol. Unless specified otherwise, explants were maintained at 5 °C in darkness for 4 d.

Dehydration and vitrification followed by LN immersion Each 2 mL sterile polypropylene cryovial (Greiner Bio-One, Frickenhausen, Germany; Cryo.s™, internal thread) was filled with ten explants in 1.5 mL of a loading solution composed of 2 M glycerol and 0.4 M sucrose in full-strength McCown Woody Plant Medium (WPM; Lloyd and McCown 1980) salts (Duchefa Biochemie) supplemented with 4.44 µM 6-benzylaminopurine (BAP) and 0.46 µM kinetin, adjusted to pH 5.8 prior to sterile filtration through a 0.2-µm membrane filter. The explants were incubated in the loading solution for 25 min at room temperature, after which the solution was removed and replaced with 2.0 mL of ice-cold modified PVS2 (Sakai *et al.* 1990) consisting of 30% (w/w) glycerol, 15% (w/w) ethylene glycol, and 15% (w/w) dimethyl sulphoxide (DMSO) supplemented with 0.4 M sucrose, 4.44 µM BAP, and 0.46 µM kinetin in full-strength WPM salts. The PVS2 was adjusted to pH 5.8 prior to sterile filtration through a 0.2-µm membrane filter, after which the cryovials were sealed and maintained on ice for 60 min before being directly immersed in LN and stored in the liquid phase in a cryogenic storage vessel for at least 25 d until rewarming.

Rewarming and recultivation For rewarming, cryovials were removed from LN and rapidly immersed in a 40 °C water bath for 1 min, followed by 10 s in another water bath at 24°C. Immediately afterwards, the vitrification solution (PVS2) was removed and the explants were rinsed three times, each with 1.5 mL of a 1.2 M sucrose solution prepared in full-strength WPM salts supplemented with 4.44 µM BAP and 0.46 µM kinetin (pH 5.8, sterile-filtered) at room temperature. The first two rinses were performed without standing time, whereas during the third rinse, the explants were left to stand for 3 min. After the final rinse, the cryovials were completely emptied onto sterile filter paper and the shoot tips were transferred onto the surface of the propagation medium in 94 × 16 mm Petri dishes with ventilation lugs (ten explants per dish, approximately 30 mL medium per Petri dish). The cultures were maintained at 22 °C in darkness for 4 d, followed by 1 wk under low light conditions (~ 10 µmol m^{-2} s^{-1}), a further week at 15 µmol m^{-2} s^{-1}, and thereafter under the standard light intensity of 20 µmol m^{-2} s^{-1}, all under a 16-h photoperiod. Eight weeks after rewarming, only viable explants, identified by their green colour and overall vitality, were transferred into 370-mL glass vessels containing the propagation medium as described above. Where these explants had already formed shoots, only the shoot tip was excised and cultivated further.

Experiment 1: Effect of explant size on post-cryopreservation performance To investigate the effect of shoot tip size on post-cryopreservation regrowth and subsequent shoot

development, two size classes of shoot tips were compared: shoot tips were prepared with a length of 1 to 2 mm or 3 to 4 mm (Supplementary Table S1). Shoot tips were hardened under four different daytime temperatures (3°C, 7 °C, 15 °C, and 20 °C) with a constant night temperature of 3°C. The hardening phase lasted 4 wk and was followed immediately by a 4-d pre-culture at 5 °C in darkness. In this experiment, five explants were placed per 94 × 16 mm Petri dish during pre-culture and per 2 mL cryovial during LN treatment. The experiment commenced on 11 November 2024 and was conducted in a single experimental repetition (RoT). For each combination of explant size and daytime temperature, the number of explants (n) was 25 for genotypes SFHb1 and E87 and 20 for genotype Gött47-38. The LN storage duration was 28 d for all variants.

Experiment 2: Effect of hardening and pre-culture duration on post-cryopreservation performance To assess how the duration of the hardening and pre-culture influenced post-cryopreservation regrowth and subsequent shoot development, four treatment variants were compared (Supplementary Table S2). Variants are denoted as wH/dP, where w is the number of weeks of hardening (H) and d the number of days of pre-culture (P). The four variants were 0wH/4dP, 2wH/4dP, 4wH/4dP, and 4wH/0dP, i.e. 0, 2, or 4 wk of hardening and either 0 or 4 d of pre-culture. Two independent RoT were conducted, initiated on 2 November 2023 and 26 February 2024, respectively. For variants involving hardening, the day/night regime was 20 °C for 8 h and 3 °C for 16 h; within each RoT, hardening generally commenced at the same time, but was occasionally delayed by a few days when required due to workload, resulting in variation of LN immersion time (Supplementary Table S2). For each RoT, 50 explants per variant were prepared, except for 0wH/4dP in RoT 1, where 60 explants were prepared for genotype SFHb1. Immersion in LN was scheduled according to the respective hardening and pre-culture durations, whereas all samples within a RoT were rewarmed on the same date.

Experiment 3: Effect of day temperature during hardening on post-cryopreservation performance The effect of the daytime hardening temperature on post-cryopreservation regrowth and subsequent shoot development was evaluated in this experiment using four treatment variants (Supplementary Table S3). In the following, these variants are referred to by their day temperature. Each variant involved a 4-wk hardening phase at a constant night temperature of 3 °C and a day temperature of either 3 °C, 7 °C, 15 °C, or 20 °C, followed by pre-culture for 4 d at 5 °C in darkness. Two independent repetitions were conducted (RoT 1 initiated on 13 June 2024; RoT 2 on 19 November 2024), each with $n = 50$ explants per variant. Immersion times in LN were 65 and 48 d for RoT 1 and 2, respectively. Due to workload constraints during explant preparation, variations in LN immersion within a variant of up to 2 d were unavoidable and are indicated in Supplementary Table S3, but all samples within a RoT were rewarmed on the same date.

Evaluation of post-cryopreservation performance Fourteen weeks after rewarming, cultures were evaluated for four parameters: regrowth, shoot multiplication, shoot length, and hyperhydricity. Regrowth was defined as the percentage of explants that produced at least one morphologically normal, propagable shoot based on the total number of explants cultured after rewarming. Shoots were classified as propagable if they possessed a well-defined shoot tip and distinct internodes, making them suitable for excision from the original explant and subsequent subculture. Explants exhibiting only callus formation without the development of an elongated shoot axis were not considered regrown. The multiplication factor was defined as the number of apical and nodal explants obtainable per rewarmed source explant. Shoot length was measured in centimetres from the apical meristem to the base of the shoot, excluding any callus, using a standard ruler. Hyperhydricity, which might also develop during propagation of regrown shoots, was scored for all explants by classifying them as affected or unaffected based on visible symptoms such as glassy, thickened tissues and malformed leaves. For statistical evaluation, however, only explants that exhibited regrowth were considered, and percentages were calculated relative to this subset.

Statistical analyses All statistical analyses were performed in R (version 4.4.2; R Core Team 2024) using RStudio (version 2024.12.1 + 563; Posit team 2025) with the packages *car* (Fox and Weisberg 2019), *emmeans* (Lenth 2025), *multcomp* (Hothorn *et al*. 2008), *multcompView* (Graves *et al*. 2024), *dplyr* (Wickham *et al*. 2023), and *ggplot2* (Wickham 2016). Analyses were conducted at the explant level; each explant represented one statistical observation, and no aggregation at the vessel level was performed. For descriptive figures, arithmetic means (continuous outcomes) or percentages (binary outcomes) are shown. For continuous outcomes (shoot length, multiplication factor), model assumptions of normally distributed and homoscedastic residuals were assessed by Shapiro–Wilk tests, Q–Q plots, and Levene's tests. As these assumptions were not met, inference was based on generalized linear models (GLMs) with a Gamma distribution and a log link. For binary outcomes (regrowth, hyperhydricity), GLMs with a binomial error distribution and logit link were fitted. Possible overdispersion was checked by comparing the Pearson chi-squared statistic with the residual degrees of freedom. All models included the fixed factors genotype, variant, and RoT, as well as their interactions. Statistical significance of model terms was assessed using likelihood-ratio chi-squared tests

(type-II ANOVA). When significant effects were detected, post hoc pairwise comparisons of estimated marginal means (EMMs) were performed using *z*-tests with Sidak correction for multiple testing, and homogeneous groups were indicated by compact letter displays (CLDs). Effect sizes with 95% confidence intervals (CIs) were reported as response-scale differences (Gamma models) or odds ratios (binomial models). All statistical tests were conducted at a significance level of $p = 0.05$.

Results

Experiment 1: Effect of explant size on post-cryopreservation performance In order to identify a suitable explant size for sycamore maple cryopreservation, in Experiment 1, two explant size classes (1 to 2 mm and 3 to 4 mm) were compared alongside four hardening day temperatures (3°C, 7 °C, 15 °C, and 20 °C) for three genotypes. Since several variants (genotype–temperature–size combinations) contained only very few or no positive observations (electronic supplementary material file Experiment1_rawdata.xlsx) and the sample sizes were relatively small, no inferential statistical analysis was performed, and the temperature factor was not further evaluated. Instead, the focus of this experiment was put on the explant size.

From all genotype–size combinations, viable shoots were obtained, indicating success in cryopreservation. However, across genotypes, regrowth rates after cryopreservation were generally much higher for smaller shoot tips (1–2 mm) than for larger ones (3–4 mm). For genotype E87 (Fig. 1), regrowth reached 56% for 1–2 mm explants but only 4% for 3–4 mm explants. A similar pattern was observed for Gött47-38 (Supplementary Fig. S1) (1–2 mm, 20%; 3–4 mm, 2%) and SFHb1 (Supplementary Fig. S2) (1–2 mm, 35%; 3–4 mm, 3%) (Table 1). Multiplication factor and shoot length also tended to be higher in cultures derived from smaller explants for SFHb1 (4.4 ± 2.4; 4.5 ± 3.4 cm) compared to larger ones (2.5 ± 2.1; 2.0 ± 1.4 cm), whereas in E87 and Gött47-38, differences between size classes were less consistent (Supplementary Table S4). Hyperhydricity was observed in only one shoot of genotype E87 derived from 1–2 mm explants (2.4%), while no cases occurred in any other genotype–size combination (Supplementary Table S5). Due to the superior post-cryopreservation performance of the smaller explants, for Experiments 2 and 3, shoot tips with a length of 1–2 mm were used.

Overall, Experiment 1 showed that explant size strongly affected post-cryopreservation performance. Across all three genotypes, smaller shoot tips (1–2 mm) consistently produced much higher regrowth and often better multiplication and shoot elongation, while hyperhydricity remained very low in all treatments. On this basis, shoot tips of 1–2 mm were selected as the standard explant size for subsequent optimization experiments.

Experiment 2: Effect of hardening and pre-culture duration on post-cryopreservation performance The aim of this experiment was to optimize the duration of the hardening and pre-culture phase. Regrowth was significantly affected

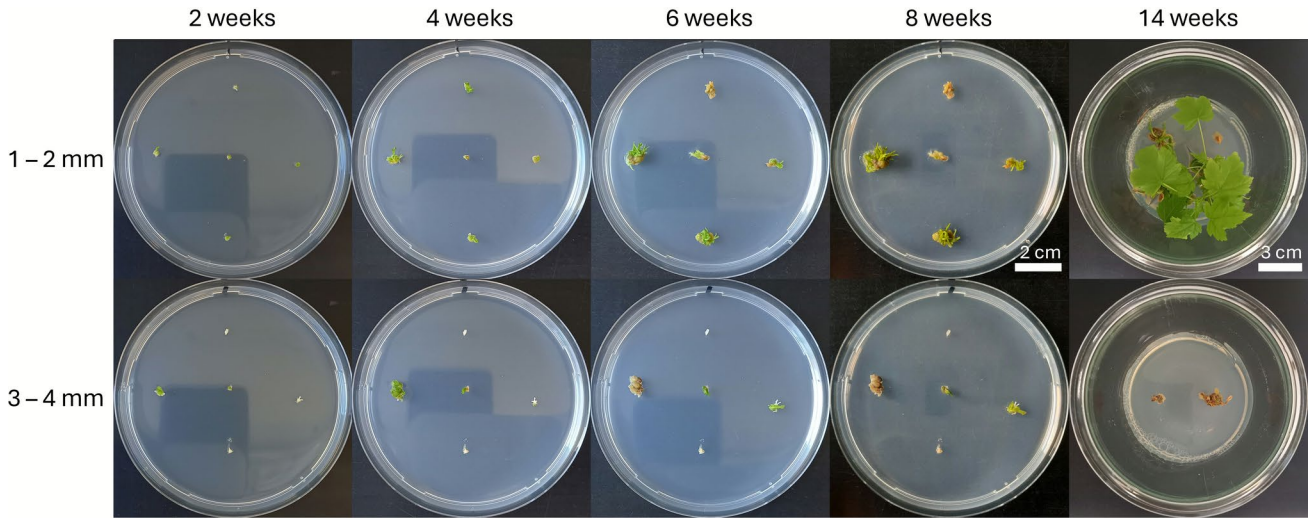

Figure 1. Shoot formation from sycamore maple (*Acer pseudoplatanus* L.) genotype E87 shoot tips of two size classes (1–2 mm and 3–4 mm) following cryopreservation (Experiment 1). Explants were hardened at 3 °C daytime/3°C night temperature for 4 wk before pre-culture and liquid nitrogen (LN) storage. Representative explants are shown at 2, 4, 6, 8, and 14 wk after rewarming. Smaller shoot tips (1–2 mm) showed higher survival and more vigorous shoot development compared with larger shoot tips (3–4 mm). *Scale bars*: 2 cm (2–8 wk) and 3 cm (14 wk).

Table 1. Regrowth of cryopreserved shoot tips of sycamore maple (*Acer pseudoplatanus* L.) 14 wk after rewarming for three genotypes and two explant size classes (Experiment 1). Values represent the proportion of explants that formed shoots, expressed as percentage with 95% confidence intervals (CI). *n* indicates the number of explants evaluated

Genotype	Explant size	n	Regrowth (%)	95% CI
E87	1–2 mm	75	56	44.1–67.3
E87	3–4 mm	75	4	1.0–12.0
Götte47-38	1–2 mm	60	20	11.2–32.7
Götte47-38	3–4 mm	60	2	0.1–10.1
SFHb1	1–2 mm	75	35	24.3–46.6
SFHb1	3–4 mm	70	3	0.5–10.9

by genotype and, to a lesser extent, by the hardening/pre-culture treatment. Likelihood-ratio tests (type-II ANOVA for GLMs) indicated highly significant main effects of genotype and variant, whereas repetition (RoT) was not significant. Significant two-way interactions were detected for the factor combinations genotype and variant, genotype and RoT, and variant and RoT (Supplementary Table S6), calling for a separate presentation of data for the two repetitions (Fig. 1).

In RoT 1, genotype E87 achieved the highest regrowth rates. Maximum regrowth occurred after 4wH/4dP (76%), followed by 2wH/4dP (64%) and 4wH/0dP (60%), while the non-hardened treatment (0wH/4dP) resulted in 58%. Götte47-38 performed consistently lower across treatments, ranging from 32 to 40% without marked differences between variants. Schussi benefitted from extended hardening, increasing from 34% in 0wH/4dP and 2wH/4dP to 54% in 4wH/4dP and 60% in 4wH/0dP (Fig. 2). SFHb1 showed the best performance in 0wH/4dP (48%) and 4wH/4dP (44%), but overall responded only weakly to prolonged hardening. Under RoT 2, the pattern shifted slightly. E87 (Supplementary Fig. S3) showed only 22% regrowth in 0wH/4dP, but increased significantly to 58–60% under 2wH/4dP, 4wH/4dP, and 4wH/0dP. Götte47-38 (photos in Supplementary Fig. S4) reached its highest values in variants 2wH/4dP and 4wH/4dP (50 and 56%), while 0wH/4dP and 4wH/0dP resulted in 36% and 24%, respectively. Schussi (photos in Supplementary Fig. S5) remained on a lower level, with maximum regrowth of 42% in 4wH/4dP and 22–38% in the other variants. SFHb1 (Fig. 3) showed rather uniform responses across most treatments (44–50%), except for 4wH/0dP, where only 30% of the explants regrew.

The shoots formed after cryopreservation elongated well and were generally of high quality, as reflected by their multiplication factor and shoot length. Both traits were significantly affected by genotype and variant, and their responses differed between repetitions, as indicated by significant interactions (Supplementary Tables S7 and S8).

Multiplication factor varied markedly among genotypes (Supplementary Fig. S6*a*). In RoT 1, the multiplication factor for E87 varied between 2.9 in 0wH/4dP and 1.7 in 4wH/0dP, while Götte47-38 and Schussi remained consistently low (1.4–2.2). In contrast, SFHb1 showed the highest multiplication factors, reaching 3.7 in 0wH/4dP and staying above 3.0 across all treatments. In RoT 2, E87 performed more evenly (multiplication factor of 2.4–3.0), whereas Götte47-38 reached 2.6 and 2.8 in 2wH/4dP and 4wH/4dP but only 1.5 in 4wH/0dP. Schussi again produced the lowest values (1.6–2.1). SFHb1 showed the strongest multiplication, with multiplication factors of 3.4 in 2wH/4dP and 2.0–2.8 in the other treatments (Supplementary Fig. S6*a*).

Shoot length was significantly affected by genotype, variant, and RoT, with additional significant interactions (Supplementary Table S8). In RoT 1, E87 shoot length was highest (2.1 cm) in 0wH/4dP compared to 1.1 cm in 4wH/0dP, while Götte47-38 and Schussi remained shorter overall (0.8–1.5 cm). SFHb1 consistently achieved the longest shoots, ranging from 2.2 to 2.6 cm across treatments. In RoT 2, E87 shoot length was moderate (1.6–2.0 cm), and Götte47-38 reached 2.1 cm in 2wH/4dP and 4wH/4dP, but only 1.3 cm in 4wH/0dP, and values were low for Schussi (1.1–1.7 cm). SFHb1 again produced the longest shoots, peaking at 2.7 cm in 2wH/4dP (Supplementary Fig. S6*b*).

Hyperhydricity was mainly determined by genotype and RoT, while treatment variant had little effect (Supplementary Table S11). Although the likelihood-ratio tests indicated significant main effects and interactions, post hoc comparisons revealed almost no significant differences between treatment means, with the exception of RoT 1 in 4wH/0dP, where Götte47-38 reached markedly higher percentages of hyperhydricity than two of the other genotypes, with up to 39% in 4wH/0dP and 29% in 4wH/4dP. In contrast, Schussi reached 25% hyperhydric explants in 0wH/4dP, but remained below 8% in the other treatments. E87 expressed only low levels of hyperhydricity (2–12%), and SFHb1 was essentially free of hyperhydricity ($<4\%$ across all treatments). In RoT 2, overall frequencies decreased. Götte47-38 reached 16% in 2wH/4dP and 14% in 4wH/4dP, while E87 remained between 2 and 13%. Schussi showed only sporadic hyperhydricity ($\leq 10\%$), and SFHb1 stayed consistently below 5% (Supplementary Fig. S7).

Across both repetitions, regrowth, multiplication, and shoot length were dominated by genotypic differences, but were also modulated by the hardening/pre-culture regime. Treatments including 2–4 wk of cold hardening in combination with a 4-d pre-culture generally supported the highest and most stable regrowth, without compromising multiplication or shoot quality, whereas non-hardened material tended to perform more variably. Hyperhydricity was largely confined to a few genotype–treatment combinations and remained low or negligible in most variants, particularly in

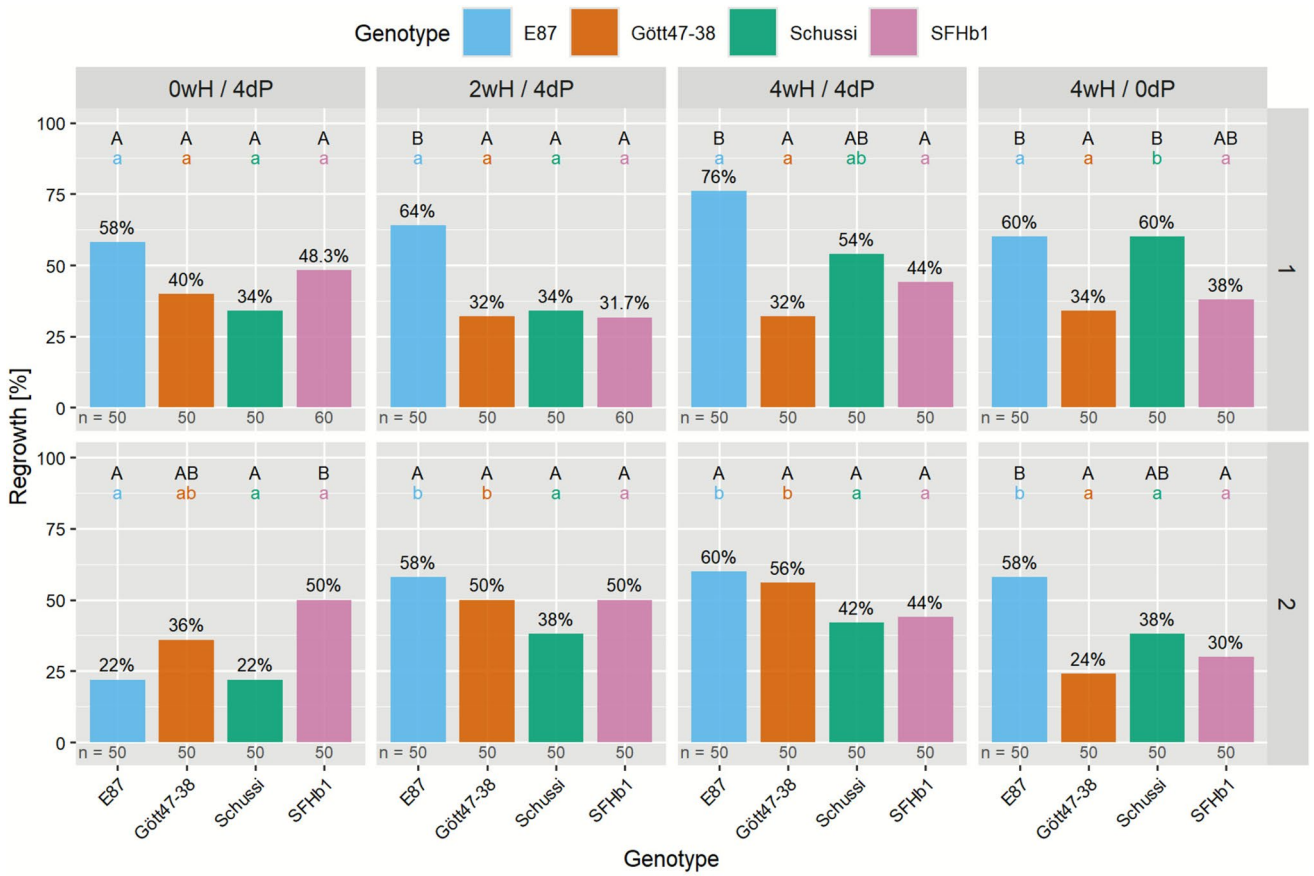

Figure 2. Regrowth after cryopreservation in the four sycamore maple (*Acer pseudoplatanus* L.) genotypes E87, Götti47-38, Schussi, and SFHb1. Percentage of explants showing regrowth 14 wk after rewarming for each genotype–treatment combination in experimental repetitions (RoT) 1 and 2 (Experiment 2). Treatments are denoted as wH/dP, with w = wk of hardening (H) and d = days of pre-culture (P). *Numbers* below the *bars* indicate the number of explants assessed (*n*). Different *uppercase letters* indicate significant differences among genotypes within variants; *lowercase letters* indicate differences among variants within genotypes (pairwise comparisons of estimated marginal means from generalized linear models using *z*-tests with Sidak correction for multiple testing, $p = 0.05$).

SFHb1. Based on these results, a hardening duration of 4 wk followed by a 4-d pre-culture was selected as the standard pre-treatment for subsequent experiments.

Experiment 3: Effect of day temperature during hardening on post-cryopreservation performance To investigate the role of the temperature during the 8 h of daytime within the hardening phase, four variants were studied, covering the range between 3 and 20°C. Regrowth was strongly influenced by genotype and RoT, whereas the *p*-value for the variant, i.e. day temperature applied during the hardening phase, was higher (Supplementary Table S10). Moreover, all two- and three-way interactions containing the factor genotype were highly significant, pointing to different reactions of the genotypes to the day temperature during hardening and between the two repetitions of the experiment (Supplementary Table S10). In RoT 2, E87 reached exceptionally high regrowth levels, with a maximum of 90% at 3 °C and another outstandingly high value of 82% at 7 °C, clearly outperforming all other genotype–temperature combinations (Fig. 4). Götti47-38 showed the steepest decline with increasing hardening day temperature in RoT 1 (52% at 3 °C to 16% at 20 °C), although it reached up to 58% at 7 °C and 20 °C in RoT 2. Schussi displayed a more variable response, peaking at 64% at 7 °C in RoT 1 and 52% at 7 and 15 °C in RoT 2. Regrowth of SFHb1 remained moderate across all temperatures (38–52%) and did not exhibit a consistent temperature-related trend. Post hoc comparisons confirmed that the highest values of E87 at 3 °C and 7 °C in RoT 2 were statistically distinct from most other genotype–temperature combinations (Fig. 4). In total, the regrowth data in Experiment 3 confirms the established protocol to reproducibly result in successful cryopreservation of various sycamore maple genotypes.

The multiplication factor recorded for the regrown shoots was strongly affected by genotype and RoT, and showed a smaller but significant effect of hardening day temperature (variant; Supplementary Table S11). SFHb1 consistently

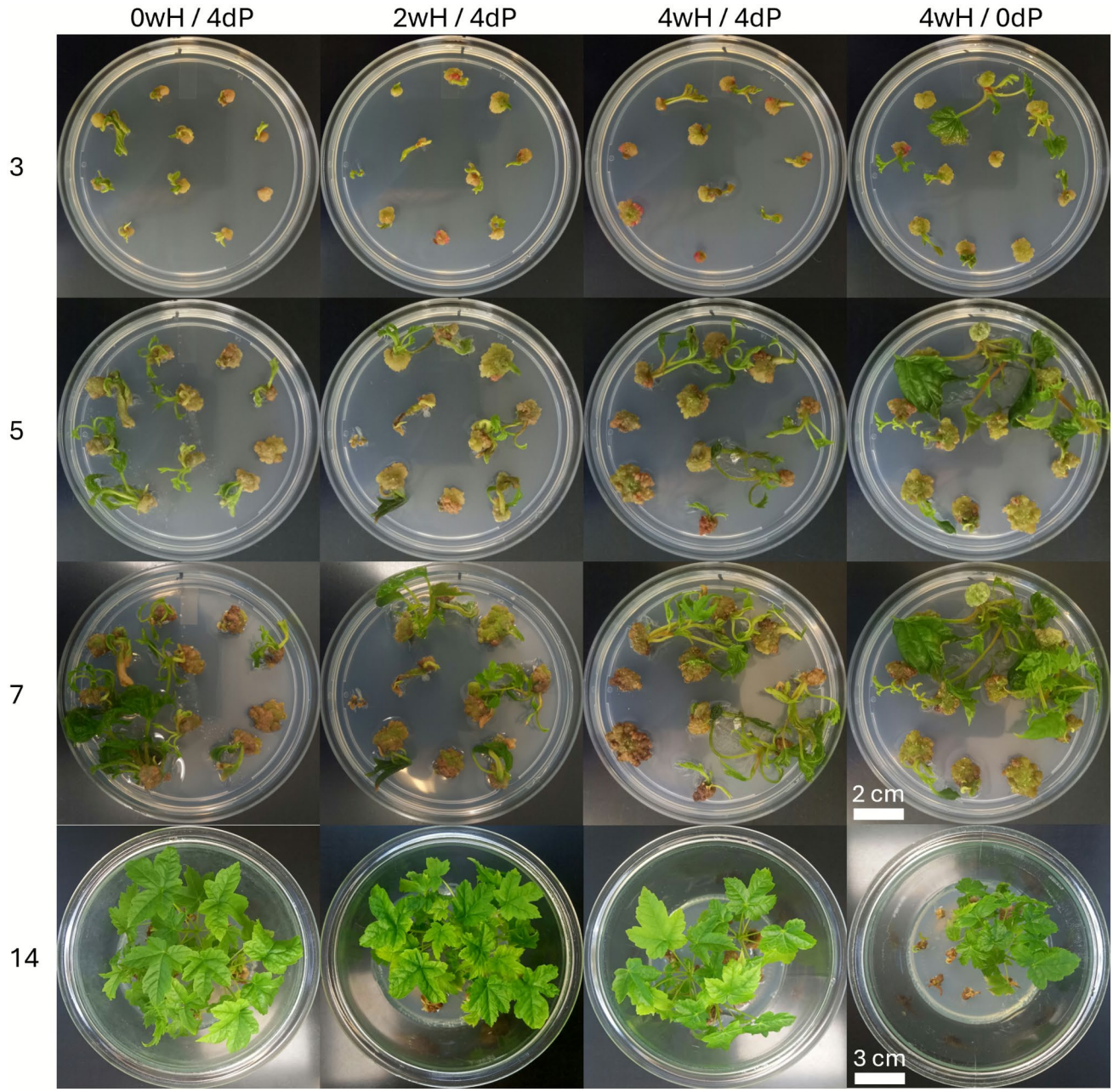

Figure 3. Representative examples of explants of sycamore maple (*Acer pseudoplatanus* L.) genotype SFHb1 (RoT 2) at 3, 5, 7, and 14 wk after rewarming, illustrating variation in regrowth intensity among the four hardening/pre-culture treatments (wH/dP, with w = wk of hardening (H) and d = days of pre-culture (P), Experiment 2). *Scale bars*: 2 cm (3–7 wk) and 3 cm (14 wk).

produced the highest values, reaching up to 4.7 for 7 °C in RoT 1 and 4.1 for 7 °C in RoT 2. E87 ranged between 2.4 and 3.3, with the maximum for 20 °C in RoT 1. Gött47-38 and Schussi generally remained below 3.3, with occasional peaks for Gött47-38 for 20 °C in RoT 1 (3.6) and for Schussi for 7 °C in RoT 1 (3.3). No general temperature trend was observed, reflecting the significant two-way interactions detected for this parameter (Supplementary Table S8 and Supplementary Fig. S6a).

The shoot length data closely resembled those of the multiplication factor, which is due to the fact that the number of nodes contributed to the multiplication factor. Shoot length was also strongly affected by genotype and RoT, with a smaller but significant effect of hardening day temperature (Supplementary Table S12). SFHb1 consistently produced the longest shoots, reaching 4.4 cm for 15 °C in RoT 2 and 4.1 cm for 7 °C in RoT 1. E87 ranged between 1.6 and 3.1 cm, with the maximum for 7 °C in RoT 2. Gött47-38 and Schussi

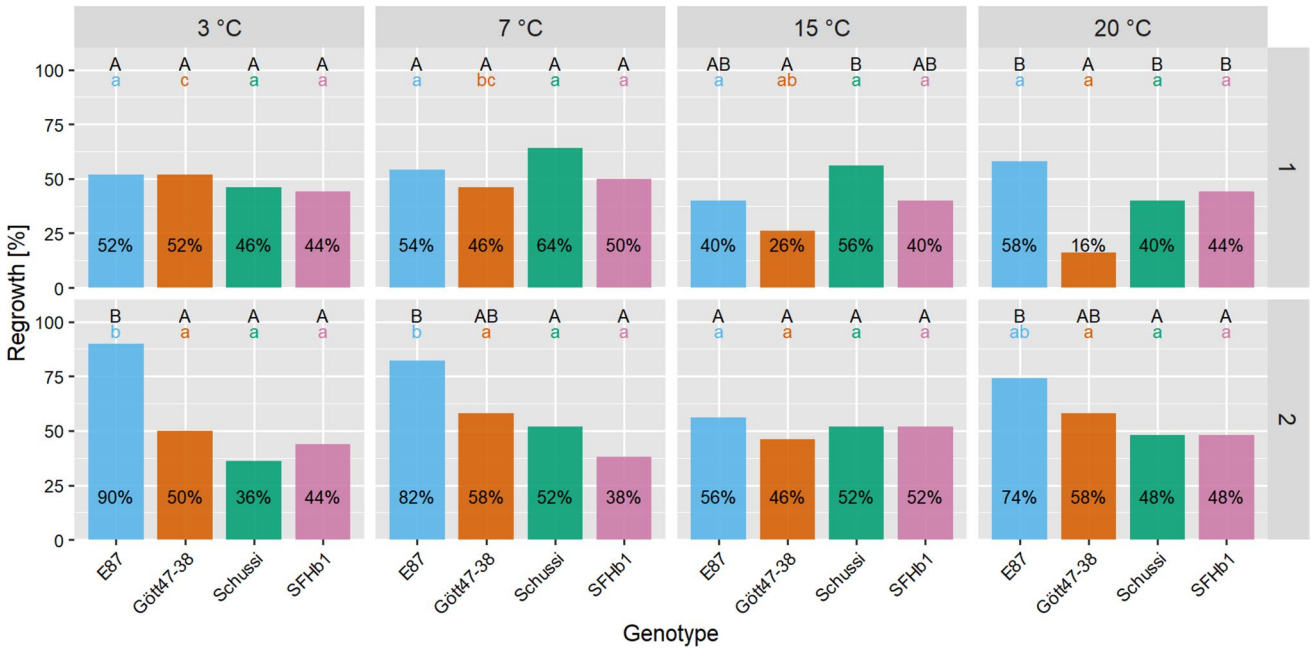

Figure 4. Regrowth after cryopreservation in the four sycamore maple (*Acer pseudoplatanus* L.) genotypes E87, Götti47-38, Schussi, and SFHb1 depending on the day temperature during 4 wk of hardening (Experiment 3). Given are percentages of explants showing regrowth 14 wk after rewarming for each genotype–treatment combination in experimental repetitions (RoT) 1 and 2. Treatments correspond to hardening at day temperatures of 3 °C, 7 °C, 15 °C, or 20 °C (night temperature 3 °C) for 4 wk, followed by 4 d of pre-culture at 5 °C in darkness. $n = 50$ per variant and RoT. Different *uppercase letters* indicate significant differences among genotypes within variants; *lowercase letters* indicate differences among variants within genotypes (pairwise comparisons of estimated marginal means from generalized linear models using *z*-tests with Sidak correction for multiple testing, $p = 0.05$).

generally remained below 2.6 cm. No consistent temperature-related pattern was evident (Supplementary Fig. S6*b*).

Hyperhydricity was significantly affected by genotype and hardening day temperature, while RoT had no significant effect (Supplementary Table S13). Significant interactions indicated a genotype-dependent reaction to the temperature treatment and the repetition. The highest proportions of hyperhydric explants occurred in SFHb1 for 20 °C under RoT 2 (27%) and in Götti47-38 for 15 °C under RoT 1 (26%). Elevated values were also observed in Götti47-38 for 3 °C under RoT 1 (23%) and RoT 2 (21%), as well as in Schussi for 3 °C under RoT 1 (20%). By contrast, hyperhydricity was absent in several combinations, such as Schussi for 15 °C (RoT 1 and 2), Götti47-38 for 20 °C under RoT 1, and E87 for 3 °C under RoT 2, and generally remained low across most treatments (< 10%) (Supplementary Fig. S9).

In summary, regrowth was mainly affected by genotype and repetition, while the effect of hardening day temperature was less consistent. While E87 reached the highest regrowth at 3 and 7 °C, for the other genotypes, no clear temperature-related effect on regrowth, multiplication factor, or shoot length was observed. Some genotype–temperature combinations showed elevated hyperhydricity, but frequencies were low in many treatments.

Discussion

Established cryopreservation protocol successfully applied to all Sycamore genotypes under investigation This study provides the first report on successful vitrification-based cryopreservation for sycamore maple shoot tips and demonstrates that genotype, hardening conditions, and explant size exert strong and interacting effects on post-thaw recovery. Across all experiments, genotype emerged as the most influential factor, confirming the genotype dependence of post-cryopreservation performance widely reported in woody species (Niino *et al*. 1992; Schoenweiss *et al*. 2005). Among the tested genotypes, E87 consistently achieved the highest regrowth, reaching up to 90% following 4 wk of cold hardening at low daytime temperatures (Fig. 4), while Götti47-38 displayed the lowest and most variable performance (Figs. 2 and 4). Likewise, in cherry and sweet cherry (*Prunus* spp.), Niino *et al*. (1997) observed large differences in survival between cultivars, and Li *et al*. (2015) documented similarly variable recovery among seven *Malus* genotypes. In *Betula pendula*, both slow-cooling and vitrification protocols revealed pronounced clonal variation (Ryynänen and Aronen 2005), and in hybrid aspen (*Populus tremula* × *P*.

tremuloides), Jokipii *et al*. (2004) demonstrated substantial genotype effects on post-thaw regrowth.

While genotype-specific optimization is valuable during protocol development, it is neither feasible nor desirable to design fully individualized procedures for large numbers of accessions in gene bank practice. Instead, the goal must be to identify broadly effective compromise conditions that achieve reliable recovery across diverse genetic backgrounds, complemented by targeted adjustments only for particularly recalcitrant or high-priority genotypes. The observed variation likely originated from physiological differences, resulting in variation regarding the efficiency of cold acclimation processes. These may include the accumulation of soluble sugars, modifications of membrane lipid composition, and the activation of antioxidant defence systems, being hallmarks of cold acclimation (Thomashow 1999; Xin and Browse 2000; Ruelland *et al*. 2009). In the context of cryopreservation, oxidative stress and the efficiency of ROS detoxification have further been highlighted as critical determinants of recovery (Ren *et al*. 2021). Thus, variation in ROS detoxification systems may explain why some genotypes responded strongly to pre-treatments while others remained largely unaffected. Despite genotypic differences in post-cryopreservation performance, all four genotypes satisfying regrowth percentages were achieved. Overall, the established protocol seems appropriate for gene bank use for which minimal regrowth rates of 40% were proposed (Reed *et al*. 1998; Popova *et al*. 2023). For additional sycamore maple genotypes, the established cryopreservation protocol can therefore be regarded as a standard framework for routine application across diverse material. In a gene bank context, larger batches of shoot tips will be cryopreserved under the optimized hardening and pre-culture conditions, while a smaller subset will be rewarmed to confirm that regrowth and shoot quality reach minimally acceptable levels for long-term storage. Genotype dependence of post-cryopreservation performance is to be expected, but only where recovery consistently remains below minimal expectations and/or pronounced stress symptoms are observed (for example, extensive browning or necrosis, pronounced chlorosis or hyperhydricity, or severe growth suppression during the first subculture after thawing), further optimization may be necessary. Future protocol refinements should primarily focus on adjustments that can be applied across multiple genotypes of the species, rather than on developing fully genotype-specific protocols. In this context, modest adjustments of key steps, including vitrification conditions, thermal transitions, and regrowth conditions, are likely the most promising options for improving overall protocol performance.

When selecting a cryopreservation strategy for *in vitro* shoot tips, three principal methods are commonly used for plant germplasm: controlled slow cooling, encapsulation–dehydration, and vitrification, from which a range of protocol variants (for example, droplet- or encapsulation-vitrification) have been derived (Panis *et al*. 2020; Bettoni *et al*. 2021; Nagel *et al*. 2024). For very small, meristematic shoot tips, vitrification protocols based on highly concentrated plant vitrification solutions, particularly PVS2 and PVS3 (Nishizawa *et al*. 1993), are widely used and often represent the most suitable strategy, as they allow rapid and relatively homogeneous dehydration and thereby minimize the risk of intracellular ice formation (Sakai and Engelmann 2007; Bettoni *et al*. 2021; Nagel *et al*. 2024). In practice, vitrification protocols using PVS2, the most frequently employed plant vitrification solution for shoot tip cryopreservation, have become a predominant approach in many plant species, and exposure time and temperature are routinely optimized for species-, explant-, and sometimes genotype-specific requirements (Sakai and Engelmann 2007; Panis *et al*. 2020; Bettoni *et al*. 2021; Nagel *et al*. 2024). These considerations provide the rationale for the widespread use of vitrification-based protocols as the primary option for *in vitro* shoot tip cryopreservation and also guided the choice of a PVS2-based approach for the shoot tips investigated in this study.

Explant size effects Our findings confirm the widely recognized importance of explant size for the success of cryopreservation in woody plants. Across all three genotypes evaluated, small *in vitro* shoot tips (1–2 mm) consistently regenerated at much higher frequencies than larger explants (3–4 mm; Table 1). Similarly, Niino *et al*. (1992) obtained successful regeneration in *Malus* and *Pyrus* using shoot tips approximately 1.5–2 mm long with a basal diameter of about 1.5 mm, although they did not experimentally assess the effect of explant size. In herbaceous species, Baek *et al*. (2003) demonstrated that explant size strongly influenced cryopreservation outcomes in garlic: very large explants (4.5 mm) performed poorly (≈42% survival, 20% regeneration), whereas smaller (1.5 mm) and medium-sized (3.0 mm) explants achieved much higher regeneration, with the latter giving the best results (≈91% regeneration).

These observations are consistent with theoretical considerations, since reduced tissue mass facilitates more uniform penetration of cryoprotectants and decreases the likelihood of intracellular ice formation (Benson 2008). They are also in line with cytological and physiological studies showing that vacuolated, differentiated cells typically fail to survive freezing and undergo lysis upon thawing, whereas small meristematic cells are more resistant (Białoskórska *et al.* 2024). In addition, oxidative stress caused by excessive ROS accumulation is a major factor reducing survival after cryopreservation (Ren *et al*. 2021), and toxic compounds released from damaged or dead cells can further compromise the recovery of sensitive explants (Popova *et al*. 2023).

While smaller explants might enhance cryoprotectant penetration, both physiological and practical limits constrain further size reduction. In *Ficus carica*, very small meristems of 0.2–0.4 mm regenerated poorly, whereas slightly larger ones of 0.5–0.7 mm showed markedly higher survival and shoot formation, likely because the latter included one or two leaf primordia and thus retained greater developmental competence (Sahraroo *et al*. 2019). Accordingly, the 1–2 mm range represents a realistic compromise between cryoprotection efficiency and tissue integrity (Baek *et al*. 2003; Reed 2008).

Role of cold hardening Cold hardening is a crucial prerequisite for the successful cryopreservation of many woody species, as it induces physiological adjustments that enhance tolerance to desiccation and freezing stress. Being also induced in autumn in nature, these include the accumulation of compatible solutes, alterations in membrane lipid composition, and the induction of protective proteins, which together stabilize cellular structures and reduce the risk of intracellular ice formation (Thomashow 1999; Xin and Browse 2000). Cold hardening was also a crucial prerequisite in our study, with the strongest benefits observed after 4 wk (Fig. 2; 4 wk of hardening followed by 4 d of pre-culture) and at daytime temperatures of 3 and 7 °C (Fig. 4), whereas higher day temperatures (15 and 20 °C) generally reduced regrowth. Comparable low-temperature hardening regimes (around 4–5°C) have been reported as prerequisites for cryopreservation in other hardwood species, including cherry and sweet cherry (*Prunus* spp.; Niino *et al*. 1997), *Malus* spp. (Li *et al*. 2015), *Betula pendula* (Ryynänen and Aronen 2005), and hybrid aspen (*Populus tremula* × *P. tremuloides*; Jokipii *et al*. 2004). However, these studies generally relied on single hardening conditions, whereas our results provide the first systematic comparison of both hardening duration (Fig. 2) and day temperature (Fig. 4), thereby identifying 3 and 7 °C as an optimal regime for the tested sycamore maple genotypes.

Post-thaw multiplication and shoot quality Multiplication rates differed among genotypes, but overall values after rewarming were comparable to those observed in non-cryopreserved shoots grown under the same culture conditions (see Table 2; Karfik *et al*. 2026). Multiplication rates of SFHb1, E87, and Schussi closely reflected those recorded during standard micropropagation. In contrast, Götт47-38 showed a wider range, with minimum values falling below those of non-cryopreserved shoots. This indicates a tendency towards reduced robustness after cryopreservation, consistent with its general sensitivity observed throughout this study (Table 2). These results demonstrate that *in vitro* shoot cultures regained multiplication capacities close to those of non-cryopreserved explants already 3 mo after thawing.

Table 2. Multiplication factors of sycamore maple genotypes (*Acer pseudoplatanus* L.) grown under identical culture conditions. Values are either derived from shoots originating from cryopreserved explants (14 wk post-cryopreservation; this study, range across variants) or from shoots derived from non-cryopreserved explants (Experiment 3, 20 µmol m^{-2} s^{-1}; Karfik *et al*. 2026)

Genotype	Shoots from cryopreserved explants (mean)	Shoots from non-cryopreserved explants (mean)
E87	2.1–3.3	2.5–3.3
Götт47-38	1.4–3.6	2.6–3.0
Schussi	1.7–3.3	1.8–2.1
SFHb1	2.6–4.7	3.4–4.0

This confirms the suitability of the established cryopreservation protocol, which safeguarded not only survival and regrowth but also the propagation potential required for culture re-establishment.

Regarding post-cryopreservation shoot quality, genotype-specific differences were observed in hyperhydricity, with E87 consistently showing very low percentages of affected explants (< 15%), whereas SFHb1 occasionally exhibited elevated levels of around 25%, and Götт47-38 was most severely affected (hyperhydricity approaching 39% in certain variants) (Supplementary Fig. S7 and S9). These patterns are in line with previous reports emphasizing the strong genotype dependence of hyperhydric responses *in vitro* (Franck *et al*. 2004; Kevers *et al*. 2004). Importantly, hyperhydricity tended to decrease during subsequent subculturing, indicating that the disorder was at least partially reversible. The phenomenon of hyperhydricity is widely recognized as a stress-induced response that is associated with oxidative stress, hormonal imbalance, impaired lignification, and insufficient apoplastic aeration (Franck *et al*. 2004; Kevers *et al*. 2004; Polivanova and Bedarev 2022). Since all steps of the cryopreservation protocol expose explants to different kinds of abiotic stress (mainly mechanical stress during preparation, osmotic stress, cold/freezing stress), this is likely the cause of the observed hyperhydricity symptoms. Popova *et al*. (2023) highlighted the potential of optimizing regrowth environments, for instance, through osmotic adjustments, modulation of light regimes, modified medium composition, or the use of exogenous additives, to mitigate these effects.

Limitations and sources of variability This study was limited to four sycamore maple genotypes, chosen to represent contrasting material from our *in vitro* collection of high-value timber genotypes. While this provided useful benchmarks for protocol evaluation, it does not capture the full intraspecific range, and broader sampling across provenances and

environments will be needed to confirm the general applicability of the protocol within the species.

Endophytic microorganisms, which are frequent in cultures of woody species and often persist despite surface disinfection (Quambusch and Winkelmann 2018), may have influenced post-thaw regrowth. Their role in cryopreservation is not fully understood. They can compromise regeneration by outgrowing from host tissues or competing for resources (Volk *et al*. 2022), yet in some cases, they appeared to support propagation success (Pham *et al*. 2018). As they may be both detrimental and beneficial, future studies should investigate their community composition and functional impact on cryopreservation outcomes.

Operator-specific effects are another potential source of variability. Subtle differences in excision precision or explant size can strongly affect post-thaw regrowth, and changes in personnel between repetitions may therefore have contributed to variation between repetitions.

Conclusions

This study establishes the first reproducible protocol for cryopreservation of sycamore maple shoot tips. Based on the outcome of the optimization experiments, the final protocol can be summarized in six consecutive steps, which are schematically illustrated in Fig. 5:

1. *Hardening*: Sycamore maple *in vitro* shoot cultures are first cultivated for 4 wk on the multiplication medium and then cold-hardened for 4 wk (3–7°C during the day, 3 °C at night) under low irradiance (approximately 15 µmol m^{-2} s^{-1}).
2. *Pre-culture*: Shoot tips (1–2 mm) are prepared from cold-hardened *in vitro* shoots and pre-cultured for 4 d at 5 °C in darkness on solid pre-culture medium containing 0.8 M glycerol.
3. *Dehydration/vitrification*: Ten shoot tips per cryovial are incubated for 25 min at room temperature in loading solution (2 M glycerol, 0.4 M sucrose in WPM with 4.44 µM BAP and 0.46 µM kinetin), after which the solution is replaced by ice-cold modified PVS2 in which explants are incubated for 60 min on ice.
4. *Freezing*: After PVS2 incubation, the cryovials are directly immersed in liquid nitrogen and stored at −196°C in the liquid phase until rewarming.
5. *Rewarming*: Cryovials are removed from liquid nitrogen and rapidly rewarmed in a 40 °C water bath for 1 min and then for 10 s in a 24 °C water bath. Immediately afterwards, the PVS2 is removed, and the explants are rinsed three times with 1.2 M sucrose solution in full-strength WPM salts with 4.44 µM BAP and 0.46 µM kinetin at room temperature, with a standing time of 3 min during the third rinse.
6. *Recultivation*: After the final rinse, shoot tips are transferred onto propagation medium in Petri dishes and kept at 22 °C in darkness for 4 d, followed by a gradual increase in light from low irradiance (~ 10 µmol m^{-2} s^{-1}) to the standard 20 µmol m^{-2} s^{-1} under a 16-h photoperiod.

Multiplication rates after recovery were comparable to non-cryopreserved controls, confirming that the protocol preserves propagation potential. A minimum regrowth threshold of 40% is proposed, while values below 30% should trigger re-testing to provide a reliable basis for collection management. The procedure defined here thus provides a practical baseline for integrating sycamore maple into *ex situ* collections of forest genetic resources.

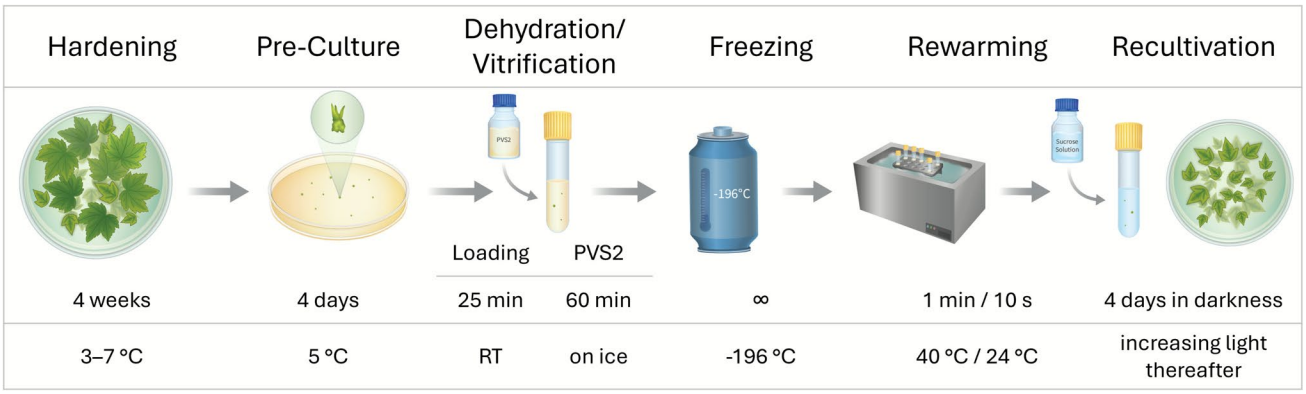

Figure 5. Optimized protocol for cryopreservation of sycamore maple (*Acer pseudoplatanus* L.) shoot tips. The procedure comprises 4 wk of cold hardening, 4 d of pre-culture, osmotic loading, vitrification with PVS2, liquid-nitrogen storage, rapid rewarming, and recultivation. Recovery was initiated with 4 d in darkness followed by a gradual increase in light intensity. Key durations and temperature conditions for each step are indicated. Graphic: Kara Perilli.

While recovery remained strongly genotype-dependent, the protocol is sufficiently robust to support long-term conservation of this ecologically and economically valuable species.

Further refinements are most likely to come from modest adjustments to the vitrification step and thermal transitions. In particular, droplet vitrification may achieve faster cooling and more uniform rewarming, especially for small shoot tips. Alternatives such as PVS3 or modified incubation times may help to balance dehydration against cytotoxic effects and could provide valuable options for sensitive genotypes. Exploring these refinements will likely broaden the applicability of the protocol and further strengthen the *ex situ* conservation of sycamore maple.

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Author contributions VK, TW, and AMD jointly developed the study design. VK conducted the experiments, analysed the data, and drafted the manuscript. TW and AMD supervised the research and provided revisions to the manuscript. All authors read and approved the final version of the manuscript.

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Data availability All raw data and statistical outputs generated during this study are provided in the electronic supplementary material files associated with this article.

Declarations

Ethics approval Not applicable.

Conflict of interest The authors declare no competing interests.

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