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Thermal analysis of cryopreserved *Hypericum perforatum* L. shoot tips: Cooling regime dependent dehydration and ice growth

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ABSTRACT

It has been well documented that unlike in vitrification during slow cooling at least two major factors, dehydration and ice crystal formation and their growth affect the survival rate of cryopreserved plant material. Presented results with shoot tips of medicinal plant species *Hypericum perforatum* show negative correlation between cooling rate and recovery after cryopreservation with a maximum of 34.4% at 0.3 °C/min. Dehydration decreases the water content in plant cells and tissues via prolonged exposure time to cryoprotective solutions and/or increased water reduction rate, both at lower cooling rates. Our results indicate no significant influence of cooling-induced dehydration on regeneration capability in samples cooled at different rates. The thermal gradients in the cryo-tube generated during the cooling process represent one of the key parameters of resulting solution's thermal behavior. Differential thermal analysis measurements confirm positive correlation between "steepness" of thermal gradients and cooling rates.

1. Introduction

The main objective of low temperature storage is to preserve unique qualities for an unlimited time period. Achievement of cryopreservation is evaluated at different levels, where survival of plant cells and tissues and ability to regenerate represent unambiguous evidence of physiological recovery. Successful recovery implies manipulation of several parameters during cryopreservation procedure resulting in numerous combinations and empirical experimentation. Therefore identification, selection and manipulation with the most critical processes towards increased viability are of great importance.

There are two major low temperature preservation approaches distinguished by the physical nature of the solidification process. Unlike vitrification where solid phase is being formed as an amorphous meta-stable state [1], two-step cooling results in crystallization and growth of ice which might eventually damage the preserved plant cells. Both crystallization and ice growth have repeatedly been shown to be cooling rate dependent [2,3] resulting in either intracellular or extracellular ice formation. Avoidance of the potential lethal damage can be achieved by lowering the crystallization temperature through reduction of the unbound freezable water content [4]. Apart from additional adjustments in pre-freezing steps decrease of the cell water content takes place during freezing as cooling induced dehydration. During this process water is removed from the cells as a result of osmotic imbalance caused by extracellular freezing [5]. However, according to the "minimum volume hypothesis" postulated by Meryman in 1968 [6], prolonged exposure to osmotically active solutes might lead to severe damage of cell compartments incompatible with life.

Despite the known inconveniences of conventional methods, controlled cooling has been used to preserve a wide range of plant germplasm. Current main application of conventional methods has been focused on undifferentiated culture systems [7,8], totipotent cell lines and embryogenic cultures [9]. Nevertheless, efficient protocols for preservation of shoot tips from vegetatively propagated crops [10], medicinal plants [11] and conifers [12] have been established.

It is evident that the success of cryopreservation strongly depends on the balance between solidification and dehydration. The optimization of cryopreservation strategies therefore resides in understanding the relation between factors which limit the conditions that contribute to the balance. In this paper we have investigated the background of cooling rate associated survival and regeneration. On the basis of measured data from differential scanning calorimetry and thermocouples we demonstrate the differences in the effects of dehydration and crystallization and assume their influence on the resulting recovery.

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2. Material and methods

2.1. Plant material and culture conditions

Seeds of *Hypericum perforatum* L. (Hypericaceae) were surface sterilized for 15 min in 1% (w/v) $AgNO_3$, washed 4–5 times in sterile deionised water and placed on the surface of basal RM medium containing Linsmaier-Skoog's salt mixture [13], Gamborg's B5 vitamins [14], 30 g/l sucrose, 2 mg/l glycine and 100 mg/l myo-inositol with the pH adjusted to 5.6 before autoclaving. Seedlings were cultured under a 16/8 h (day/night) photoperiod and irradiance of 43.34 μmol m^{-2} s^{-1} at 23 °C and 40% relative humidity. After 21–28 days the shoot tips with one pair of the upper leaves were excised and transferred to 25 ml liquid RM basal medium.

2.1.1. Preculture

Shoot tips isolated from *in vitro* grown plants were precultured in 10 ml liquid RM basal medium (pH 5.6) supplemented with 0.5 mg/l benzylaminopurine (BAP) and one of the two preculture additives, 0.3 M mannitol or 0.076×10^{-6} M ± abscisic acid (ABA) (Fluka Chemie, AG) under the same physical culture conditions as above for either 7 days for mannitol or 10 days for ABA pretreatment [11].

2.1.2. Cryoprotection

90 shoot tips were divided into 3 sterile cryotubes (2.0 ml Sarsted) containing 1.5 ml cryoprotective solution comprising of 10% (w/v) glycerol, 10% (w/v) dimethyl sulfoxide (DMSO) and 0.58 M sucrose; pH = 5.8. After 60 min equilibration on ice samples were subjected to controlled cooling process.

2.1.3. Freezing and thawing

Freezing took place in a PLANER 300&500 series controlled freezer (Planer PLC, UK) with the following program: +3.0 °C starting temperature; 0.5 °C/min to −6.0 °C; variable velocities to −40.0 °C; 10 °C/min to −80 °C; equilibration at −80 °C for 10 min. 9 different cooling rates 2.0; 1.5; 1.0; 0.8; 0.6; 0.4; 0.3; 0.2; 0.1 °C/min were applied as variable. After equilibration the samples were immersed directly in liquid nitrogen (LN) and stored for at least 2 days. Thawing was performed in a water bath warmed to 50 °C for 2 min, followed by 1 h washing in basal liquid RM medium in the dark.

2.1.4. Recovery assessment

Shoot tips were transferred onto 25 ml semi-solid RM medium containing 0.5 mg/l BAP and cultured for 2 weeks in the dark, followed by the same light and temperature conditions as described above. Recovery was determined as percentage of shoot tips capable of regeneration from the initial number frozen. Observations were completed 46 days after thawing.

The pretreatment steps, cooling procedure and regeneration were performed in different seasons, vernal (March) and autumnal (October), each in two replicates.

2.2. Thermal analyses

2.2.1. Differential scanning calorimetry (DSC)

DSC measurements were performed with differential scanning calorimeter (TA Instruments, Inc., New Castle, DE, USA) in two repetitions. *H. perforatum* L. shoot tips were divided into two sample sets both transferred to 1.5 ml cryoprotective solution and placed on ice for either 1 or 4 h long equilibration. Every half hour in course of 4 h equilibration approximately 8–10 mg of shoot tips were dried off the excessive cryoprotective solution covering the shoot tips, hermetically sealed in DSC aluminum hermetic pans and measured. Second set of samples was subjected to 1 h ice treatment followed by controlled cooling in the programmable freezer with 5 cooling rates: 2.0; 1.5; 1.0; 0.5; 0.1 °C/min for either 5 min or to the final temperature of −5 °C. For the DSC measurements the samples were processed as described above. Identical program was applied with 10 °C/min rate in cooling and heating cycle and equilibration step at −140 °C. Measured data were evaluated with Universal Analysis 2000 software version 4.1D build 4.1.0.16 (TA Instruments-Waters LLC, New Castle, DE, USA).

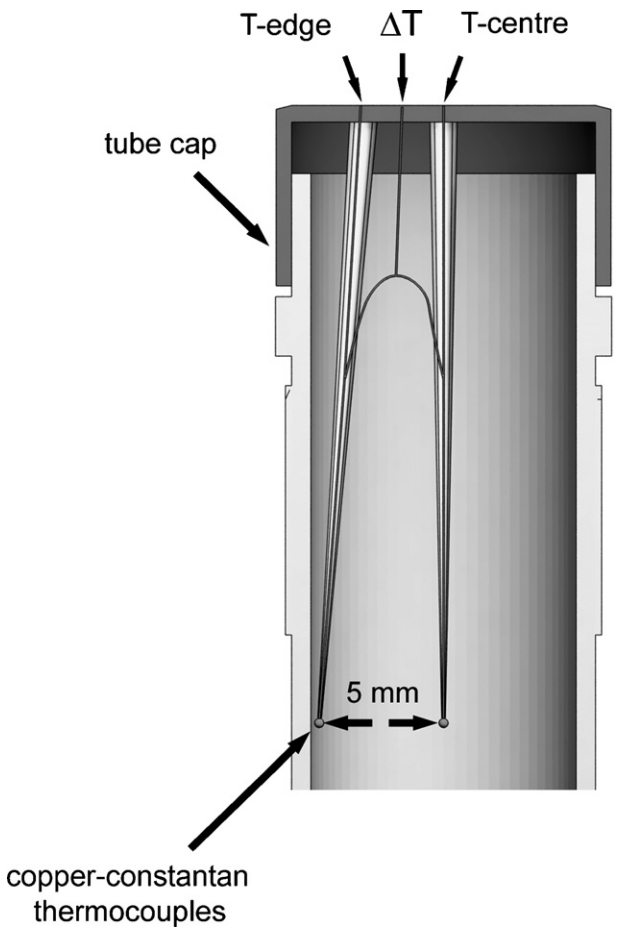

Fig. 1. Scheme of differential thermal analysis measurement apparatus comprising of two copper-constantan thermocouples in centre to edge alignment enabling to measure both actual temperatures in centre (*T-centre*) and edge (*T-edge*) and the thermal difference (ΔT) over the distance of 5 mm directly in the tube.

2.2.2. Thermocouples

Two copper-constantan thermocouples were attached to cryotube cap and positioned in the centre/edge alignment (Fig. 1). Thermocouples were calibrated over the range of 0 to −196 °C in water/ice mixture and LN. Samples consisting of 1.5 ml pure cryoprotective solution were exposed to differential thermal analysis over a range of 5 cooling rates (10.0; 5.0; 1.0; 0.5; 0.1 °C/min) in a programmable freezer. Exact temperatures and centre/edge differences were measured and collected with Control Web 2000 software build 4.1.1759.1 (Moravian Instruments, Inc., CZ). All thermal analyses were conducted in two replicates.

2.3. Statistics

Statistical analysis was performed using STATGRAPHICS version 5.0 after proving the homogeneity of variance (Cochran's test), differences between samples were evaluated by the analysis of variance ANOVA test. All tests were conducted at the 0.05 level

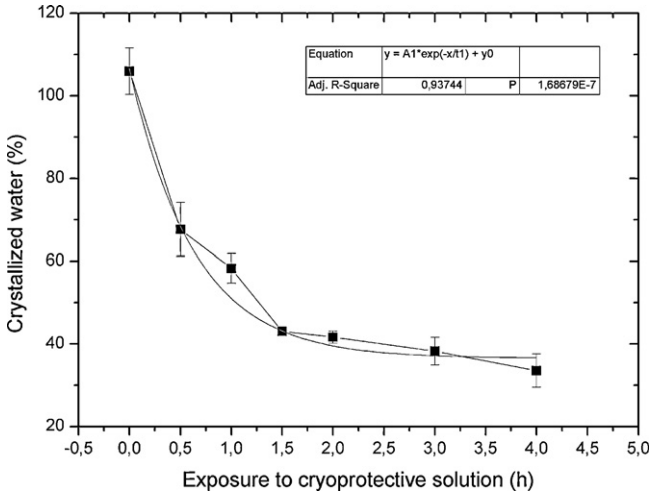

Fig. 2. Effect of cryoprotective solution on freezable water content in plant shoot tips.

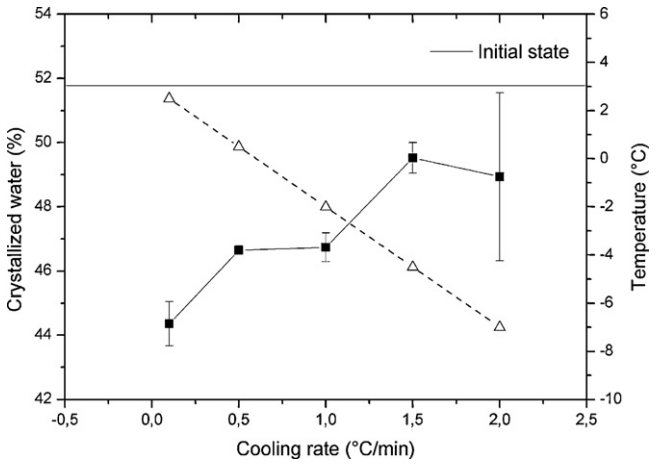

Fig. 4. Dehydration of plant cells cooled during the constant time of 5 min (-■-) from the initial hydration state after 60 min equilibration on ice. Differences in reached temperature (-△-) point to higher water diffusion at lower cooling rates.

of significance.

3. Results

3.1. Effect of cryoprotective solution on water content in the shoot tips

Shoot tips were subjected to cryoprotection in 1.5 ml of cryoprotective solution and placed on ice for 4 h. Every 30 min the shoot tips were processed for DSC assays. The results of DSC measurements show that prolonged exposure of shoot tips causes exponential decrease of the water content able to crystallize. After 1 h exposure the water content decreased to 51.8% (3.05 ± 0.057 g water/g DW) and continued to decline exponentially until stabilized after 3 hour leaving 36.8% of freezable water (2.44 ± 0.084 g water/g DW) in the tissues (Fig. 2).

3.2. Effect of cooling rate on water content in the shoot tips

After 60 min equilibration on ice the samples were placed in the controlled freezer and cooled with 5 cooling rates (2.0; 1.5; 1.0; 0.5; 0.1 °C/min) for either 5 min or to the final temperature of −5 °C. With the application of temperature as a constant (Fig. 3) the water content significantly declined to 38.6% (2.36 ± 0.082 g water/g DW; $P = 0.043$) when cooled with the lowest rate. Similar results were obtained with the time (5 min) as constant (Fig. 4) where the overall freezable water content decreased to 44.4% (2.71 ± 0.094 g water/g DW; $P = 0.048$). The highest difference in the water content reduction between cooling rates was calculated as 7.4% (0.45 ± 0.016 g water/g DW) and 5.1% (0.32 ± 0.01 g water/g DW) for constant cooling time and constant final temperature, respectively.

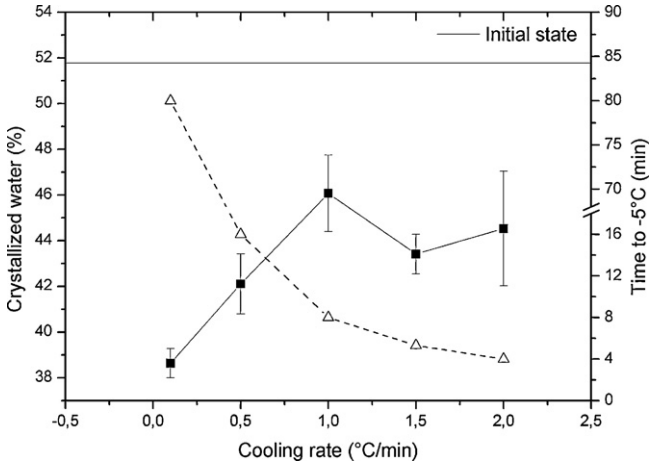

Fig. 3. Dehydration of plant cells cooled to the constant temperature of −5 °C (-■-) from the initial hydration state after 60 min equilibration on ice. Differences in time to reach −5 °C (-△-) indicate prolonged exposure to cryoprotective solution.

3.3. Effect of cooling rate on thermal gradients in the specimen

Thermal gradients were measured within specifically adjusted cryovial containing two copper-constantan thermocouples measuring thermal differences in the outer and inner compartment of the cryotube. Samples of pure cryoprotective solution were subjected to differential thermal analyses over a range of cooling rates. Recorded data (Fig. 5) revealed generation of thermal gradients along the diameter of the cryotube dependent upon the cooling rate. As indicated in Table 1 faster cooling rates resulted in larger temperature differences in the liquid phase of the cryoprotective solution reaching 4.4 ± 0.07 °C over the distance of 5 mm. During the phase transition we have observed an increase in temperature as a result of latent heat release. Regardless the overall temper-

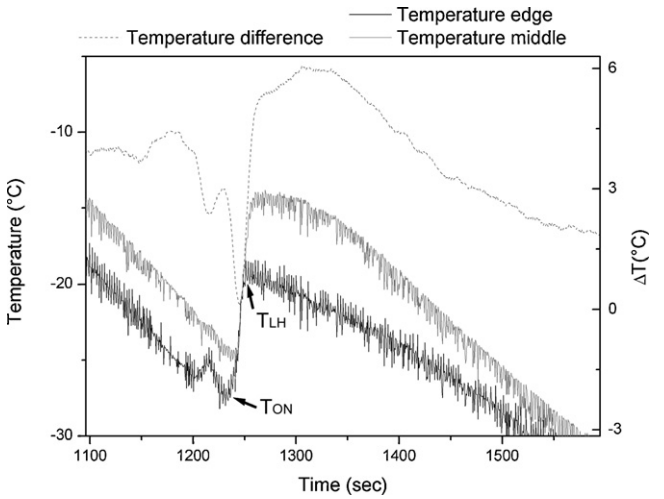

Fig. 5. Results of differential thermal analyses (10 °C/min). T_{ON}: phase transition onset temperature, T_{LH}: temperature after latent heat release

Table 1
Effect of cooling rate on cryoprotective solution's thermal behavior.

Cooling rate [°C/min]	ΔT before PT [°C ± SD]	T_{ON} [°C ± SD]	T_{LH}–T_{ON} [°C ± SD]	PT heating rate [°C/s ± SD]	Time to freeze [min ± SD][a]
10.0	4.4 ± 0.07	−27.3 ± 0.02	7.8 ± 0.03	1.6 ± 0.23	3.0 ± 0.002
5.0	4.2 ± 0.02	−26.3 ± 0.01	7.0 ± 0.01	1.3 ± 0.29	5.7 ± 0.001
1.0	0.7 ± 0.09	−21.1 ± 0.04	5.7 ± 0.03	0.2 ± 0.02	24.1 ± 0.04
0.5	0.8 ± 0.12	−25.0 ± 0.01	7.3 ± 0.07	0.017 ± 0.003	56.1 ± 0.02
0.1	0.5 ± 0.05	−22.2 ± 0.01	7.7 ± 0.11	0.016 ± 0.001	252.5 ± 0.14

PT: phase transition, T: temperature, SD: standard deviation, T_{ON}: phase transition onset temperature, T_{LH}: temperature after latent heat release.
[a] From the initial temperature of +3 °C.

Table 2
Effect of cooling rate on the recovery of plant material cryopreserved in vernal season (March) showing significant influence of cooling velocity despite the pretreatment applied.

Preculture agent	Recovery rate [%]								
	Cooling rate [°C/min]								
	2.0	1.5	1.0	0.8	0.6	0.4	0.3	0.2	0.1
0.076 μM ABA	0.00[a]	6.25[ab]	6.67[ab]	11.11[ab]	3.75[ab]	3.75[abc]	12.22[b]	21.54[c]	24.38[c]
0.3 M manitol	0.00[a]	3.75[ab]	6.88[ab]	0.00[ab]	10.00[ab]	27.50[abc]	34.44[b]	28.46[c]	33.33[c]

[a,b,c]Statistical significance between homogenous groups on $\alpha = 0.05$.

ature increase by the latent heat release has not been shown to be cooling regime dependent, considerable variation was recorded amongst heating rates.

3.4. Effect of cooling rate on recovery of cryopreserved shoot tips

10 shoot tips per genotype were subjected to pretreatment/cryoprotection followed by controlled freezing with 9 different cooling rates and storage in LN. Recovery rate was determined after 46 days as a percentage of shoot tips that regenerated into whole plantlets. The mean recovery rate varied between zero and 34.4% depending on the cooling rate (Table 2). Lowering the cooling rate resulted in recovery rate increase. Negative correlations were calculated for both, 0.076×10^{-6} M ABA ($R = 0.7$; $P = 0.036$) and 0.3 M mannitol ($R = 0.82$; $P = 0.007$) preculture conditions. Furthermore, shoot tips cooled at low rates exhibited minor tissue disintegration. No significant differences in recovery were detected between these pretreatments. In contrast, seasonal effect on shoot tips recovery showed distinct variation. The shoot tips frozen and recovered in spring exhibited 3.88 ($P = 0.004$) and 3.96 ($P = 0.0001$) times higher viability for ABA and mannitol treatments, respectively compared to autumnal experiment (Table 3).

4. Discussion

The introduction of low temperature preservation concept caused a major transformation in the long term conservation strategies. Newly designed protocols are being released and successfully implemented thereby expanding the list of preserved plant species. Among them two methodological categories can be distinguished. Slow cooling is considered as a classical freezing method where crystallization and ice growth along with osmotic dehydration were proposed to have essential impact on the preserved material [15]. Neither of these processes is present in vitrification based protocols, where high cooling rates reduce the osmotic efflux of the cell water [16] and increased viscosity inhibits molecular rearrangements of water into crystalline lattice [17]. Consequent meta-stable glassy state prohibits potential mechanical damaging by ice crystal growth and respective over-dehydration of the preserved plant material. It is one of the reasons that vitrification based protocols became the most applicable in plant cryopreservation strategies.

Despite the known difficulties conventional methods have been used for preservation of a large number of plant species and explant types. Amongst them *H. perforatum* L. as a natural source of valuable bioactive substances has been cryopreserved with both, slow cooling and vitrification [11,18,19]. Assessments at physiological, biochemical and molecular levels have been performed indicating no significant alterations in the studied profiles. Furthermore, exposure to cold has been shown to contribute to the higher content of naphtodianthrones as the most valuable pigments used in photodynamic therapy and diagnostics of cancer [19]. The mean recovery however, remained low. Several variables have been modified in order to increase the insufficient recovery and regeneration rates. Alterations in cryoprotectant compositions and time of exposure resulted in higher recoveries, however not exceeding 21%. The recently published paper on vitrification of *H. perforatum* L. presented average recovery rates of 26% with the maximum of 63% [19].

There are at least two major factors in the cooling process contributing to the survival of cryopreserved plant cells and tissues. Osmotic injury and/or mechanical damage are often the main cause behind the reduced recoveries. Their dependency upon the cool-

Table 3
Seasonal effect on the recovery of plant shoot tips indicating a significant influence of seasonal biorhythms.

Cooling rate [°C/min]	Recovery rate [%]			
	March		October	
	0.076 μM ABA	0.3 M manitol	0.076 μM ABA	0.3 M manitol
0.3	12.22[b]	34.44[c]	2.50[a]	11.67[a]
0.2	21.54[b]	28.46[c]	6.36[a]	11.67[a]
0.1	24.38[b]	32.50[c]	7.27[a]	5.00[a]

[a,b,c]Statistical significance between homogenous groups on $\alpha = 0.05$.

ing rate has been previously described by Mazur in the two-factor hypothesis, where slow cooling rates generate high intracellular concentration of solutes causing osmotic damage whereas high cooling rates result in formation of intracellular ice [20]. As a consequence the highest survival could be achieved by cooling rate sufficiently slow to avoid intracellular ice formation thus preventing the solute effect injury. Low temperature induced dehydration and ice crystal formation and growth are therefore considered to be the key issues in plant thermal preservation.

Impact of each of these processes is influenced by the content of freezable water. The optimum volume is largely achieved empirically by the exposure of plant material to various cryoprotectants for prolonged time periods. The utilization of differential scanning calorimetry (DSC) methods enabled measurements of freezable water amounts and water transport with higher precision [21]. In order to determine the extent of low temperature induced dehydration on the survival we performed several DSC assays. The experimental data output indicated an exponential decrease of freezable water content in *H. perforatum* L. shoot tips during pre-cryogenic treatment that has stabilized after 3 h exposure to cryoprotective solution at a level of 36.8% (2.44 ± 0.084 g water/g DW). The pre-freezing hydration level, usually achieved after 60 min of cryoprotective treatment has been assessed at 51.8% (3.05 ± 0.057 g water/g DW). Presuming that the optimum moisture content when preserving multicellular structures such as embryos has been considered 0.3 g water/g DW for various plant species [22], the amount of freezable water in *H. perforatum* shoot tips even after 4 h of cryoprotective dehydration remained 8 times higher. Apart from this experimentally adjustable pre-cryogenic dehydration, further decrease of water content takes place during the cooling process. It was observed that variation of cooling rates had two closely related effects contributing to dehydration of living cells. At low cooling rates, plant material exhibited substantially longer exposure to cryoprotectants giving sufficient time for water efflux from the cells. Secondly, the temperature difference over time was lower resulting in higher water diffusion. Both effects have been experimentally proven by the significant reduction of water content at the lowest cooling rate. Similar presumptions on the cooling rate dependent water outflow have already been proposed by Dumont et al. [16] as an explanation of decrease in *Saccharomyces cerevisiae* survival frozen with intermediate cooling rates (200 °C/min). Although considerable differences in water content of *H. perforatum* shoot tips were recorded between cooling rates, the highest reduction has been calculated as 7.4% (0.45 ± 0.016 g water/g DW). There are reports on substantial improvement of survival after moisture reduction of less than 0.1 g water/g DW [23,24]. These results come from experiments conducted on sufficiently dried seeds with initial moisture contents of 0.5 g water/g DW. On the other hand highly hydrated embryos of oil palm with water contents comparable to those found in *H. perforatum* shoot tips had to be exposed to dehydration for over 12 h resulting in the reduction of more than 2.0 g water/g DW in order to significantly increase the survival after LN treatment [25]. Therefore we assume that within the relatively high initial hydration level of *H. perforatum* L. shoot tips the water reduction differences generated by cooling rates are not the major contributors to cooling rate dependent survival.

During slow freezing the cooling rate is considered to play a vital role in affecting survival of cells and tissues. Despite the "solution effect" at low cooling rates it also influences the crystallization through morphology of the ice crystals [26] and their growth. Ice growth in an aqueous solution is generally limited by at least the diffusion of heat and solute away from the ice-solution interface and the interfacial tension between the ice and the solution [27]. The diffusion processes are driven by the temperature gradients dependent upon cooling rate and thermal conductivity of sample materials. It was observed in our experimental examination that higher cooling velocities generated thermal differences reaching 4.4 ± 0.07 °C over 5 mm diameter. We presume that creation of large temperature gradients by high cooling rates is responsible for the formation of thermodynamically imbalanced environment. In such environment centre to edge heat flows prohibit the nucleation of the outer edge compartment that becomes more supercooled. This presumption is supported by findings of Milón and Braga [28] who pointed out that pure or multicomponent materials are nucleated below their freezing point when cooled in an enclosed container. Furthermore, they have described different types of freezing processes depending on the coolant temperature and thermal conductivity of the container, both of which affect the thermal distribution in the container. Applying high cooling velocities the energy level of supercooled liquid area becomes so low that when nucleation occurs, phase change happens quickly and immediately releasing more latent heat compared to lower cooling rates. Despite the overall increase in temperature by the latent heat release was not shown to be dependent on the cooling regime, considerable variation was recorded amongst heating rates. Measured data on phase transition heating velocities depicting larger temperature shifts in time period for faster cooling rates could indicate crystallization of larger volumes/time period therefore faster progression of ice front which is consistent with the supercooling and instantaneous process described by Milón and Braga [28]. Furthermore, supercooling event is strongly connected with creation and fast progression of dendritic ice [29] raising the possibility of mechanical damage of tissues which is concurrent with the morphological examination of the fastest cooled *H. perforatum* shoot tips that were observed to have substantial loss in tissue integrity. The tissue ruptures might have been caused by rapid growth of needle-like external ice observed by Ishiguro and Rubinsky [2] as a result of excessive supercooling. As a consequence the resultant recovery of *H. perforatum* decreased with the application of higher cooling rates. A negative correlation between cooling rate and recovery has been calculated for both, ABA ($R = 0.7$, $P = 0.036$) and mannitol ($R = 0.82$, $P = 0.007$) pretreatments. Furthermore the overall recovery increased over 1.5 ± 0.26 times after reduction of cooling rate compared to previous methods applied. Based on the insignificant difference of regeneration capability between pretreatments despite the cooling rate used, the contribution of pretreatment to the overall recovery has been considered as minor.

Apart from externally adjustable parameters of the cryopreservation procedure as pre-culture (pre-cryogenic) additive compositions, time exposures, cooling and thawing rates a large portion of influence resides in biological processes of the preserved tissue. Despite the *H. perforatum* shoot tips have been cultured in defined *in vitro* conditions for 24 months, reduction of recovery rate for all tested samples was observed in the autumn period indicating seasonal biorhythm. Although the recovery decreased 3.88 ($P = 0.004$) and 3.96 ($P = 0.0001$) times when cryopreserved in October for ABA and mannitol treatments respectively, the influence of the freezing velocities remained comparable. This classifies the cooling rate as one of the major factors affecting the survival in cryopreservation strategies. Nevertheless, it can be concluded that cooling rate affects survival of cryopreserved shoot tips of *H. perforatum* L. through the crystallization and ice growth rather than dehydration. At lower cooling rates the temperature distribution in the cryotube during cooling is more homogenous, characterized by small thermal gradients. Although the following phase transition originates from a supercooled liquid, heat release rates suggest for a slower and non-invasive ice growth preserving the tissue integrity and function. These aspects should be considered while designing conventional cryopreservation experiments in order to avoid unwanted reduction of survival in cryopreserved plant material.

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