Plant Omics Journal POJ

POJ 5(3):291-297 (2012) ISSN:1836-3644

Genotype-dependent response of St. Johǹ`s wort (*Hypericum perforatum* L.) shoot tips to cryogenic treatment: Effect of pre-culture conditions on post-thaw recovery

Linda Petijová, Matúš Skyba, Eva Čellárová*

Institute of Biology and Ecology, Faculty of Science, P. J. Šafárik University in Košice, Mánesova 23, 041 54 Košice, Slovakia

***Corresponding author: eva.cellarova@upjs.sk**

Abstract

Cryopreservation of medicinal plants is an important tool for restoration of their potential to synthesize valuable secondary metabolites. Establishment of an efficient protocol requires detailed understanding of species/genotype-specific responses to changing external variables. The aim of this work was to study the genotype-dependent response of *Hypericum perforatum* L. shoot tips to different pre-cryogenic conditions. Shoot apices isolated from plants growing on the hormone-free medium and from clusters induced by addition of benzylaminopurine, were subjected to pre-culture using two agents (0.3M sucrose and 0.076µM abscisic acid) prior to cryopreservation. Although the genotypes represented the seed progeny of one mother plant, the results showed their different capability to withstand cryogenic treatment. The differences were apparent especially in case of plants cultivated on the hormone-free medium. The ability to recover depended also on the pre-culture agent, duration of the pre-culture period and under certain conditions also on interaction between benzylaminopurine and abscisic acid in the medium. The results are illustrated by anatomical alterations of the meristematic zones and adjacent leaf primordia. Long term exposure of cultures to benzylaminopurine resulted in the delay of abscisic acid effect during pre-culture step and thus impaired regeneration capability of shoot apices after cryopreservation.

Keywords: *Hypericum perforatum* L., cryopreservation, abscisic acid, sucrose, benzylaminopurine.

Abbreviations: ABA-abscisic acid, BAP-benzylaminopurine, LN-liquid nitrogen, LS-loading solution, PVS-plant vitrification solution.

Introduction

Over the last few decades, use of cryogenic temperatures has become an efficient tool for long-term preservation of phytodiversity or unique plant features (Pence, 1999; Yoshimatsu et al., 2000). Moreover, in biotechnological terms, cryopreservation of plant cells and tissues enables their ability to produce various bioactive metabolites to be maintained or even improved. The importance of *Hypericum perforatum* L. (St. John's Wort) as a model for an extensive *Hypericum* genus lies in the anti-depressive, anti-cancer, anti-viral and anti-inflammatory properties of its biologically-active secondary metabolites such as naphtodianthrones (hypericins) and phloroglucinols. Red photosensitive pigment, hypericin and its derivatives have been successfully applied for photodynamic diagnostics and therapy of cancer. These features rate *H. perforatum* among the potential candidates for long-term storage in a cryo-environment. Furthermore, the ability of cryopreserved *H. perforatum* plants to produce hypericins remained unaltered or slightly increased (Skyba et al., 2010).

Since the major role of cryobanks as large depositories of plant genetic reserves lies in their accession, accumulation and long-term storage, it is necessary to establish efficient cryopreservation protocols for particular species and/or explants. These protocols could be established either by mostly empirical modification of external variables, e.g. pre-culture/cryoprotection additives and exposure times, and/or on the basis of understanding the fundamental factors decisive for pre-determining the plants to withstand cryogenic temperatures. Among them, physiological status prior to cryogenic treatment is considered as one of the most critical which could affect the post-thaw regeneration ability of cryopreserved material. Furthermore, pre-culture conditions should be carefully manipulated in terms of possible interactions with some culture medium constituents which might be essential for cell proliferation (Havlová et al., 2008). In several cases, benzylaminopurine (BAP) added to culture media could interfere with abscisic acid (ABA) used during pre-conditioning of plants prior to cryopreservation (Tran et al., 2007).

In this study we have focused on some genetic and physiological aspects of *H. perforatum* cryopreservation as follows: (1) genotypic variation in the ability of shoot tips to withstand the low-temperature associated stresses under the same pre-cryogenic conditions; (2) possible interaction between long-term exposure of shoot tips to cytokinin (BAP) in the culture medium and ABA as a pre-culture additive; (3) anatomical differences of shoot apices subjected to cryopreservation after short/long exposure to BAP.

Results

Effect of genotype on post-cryogenic recovery

Plant material used in the experiments consisted of *H. perforatum* shoot tips isolated from individually-growing plants on the hormone-free RM medium and those derived from clusters differentiated during long-term continuous cultivation on the medium supplemented with cytokinin BAP (RMB medium). Both sets of samples were subjected to pre-cryogenic treatment followed by vitrification. The samples were pre-treated in the liquid RMB medium supplemented with 0.3M sucrose or 0.076µM ABA, each with three different exposure times. Recovery rate of sucrose-treated shoot tips isolated from individual plants varied from 3.3 to 50 %. The variation range indicated different abilities of individual genotypes to recover as proved by one-way ANOVA ($P \leq 0.05$) (Table 1A). The genotypes 29/7/5 and 34/7/1 showed evidently higher tolerance to cryogenic treatment than the others. Comparable variation range (from 3.9 to 62 %) with less significant inter-genotypic differences was observed in response to ABA pre-treatment (Table 1B). The results showed dependence of the mean recovery rates on the pre-treatment period. Moreover, the majority of genotypes achieved their maximum recovery after the longest exposure to either sucrose or ABA. Comparison of the average recovery rates revealed that the capability of shoot apices to regrow depended to a certain degree on the pre-culture time as proved by statistical evaluation using Mann-Whitney U test, but mostly on the genetic predisposition of particular *H. perforatum* genotypes. The ability of shoot apices isolated from clusters differentiated on BAP-enriched medium to recover after pre-treatment with 0.3M sucrose ranged between 2.9 and 40 % (Table 2A). Whereas 16 hour long sucrose treatment was evidently insufficient, prolongation of pre-culture time to either 24 or 48 hours led to improvement of the recovery rates. Post-cryopreservation recovery and regrowth of explants pre-cultured with ABA was comparable with the previous ones and varied from 2.9 to 37.5 % (Table 2B). Although statistical tests did not confirm any significant differences between different exposure times to ABA, the average recovery rates showed almost linear increasing tendency. In contrast, five genotypes responded to cryopreservation with improved recovery rates after 3 day long ABA pre-treatment.

Effect of ABA-BAP interaction on the recovery after cryopreservation

All genotypes used in the experiments showed improved capability to regrow after cryopreservation depending on the presence/absence of BAP in the culture medium during long-term cultivation (Fig. 1). Differences between average recovery rates of shoot tips isolated from plants growing on the hormone-free-medium and from BAP-induced clusters were evident and statistically proved especially after ABA pre-treated samples. To discover whether decreased regeneration rate occurred as a consequence of short and/or long-term exposure of shoot apices to BAP and its predicted interaction with ABA during pre-culture resulting in a delay of its protective influence, both sets of plants were subjected to vitrification after 10 days of 0.076µM ABA pre-treatment in liquid medium without BAP. The recovery rates of shoot tips isolated from both individually-growing plants and clusters were comparable (Table 3). However, recovery rates of shoot tips which were not exposed to BAP at all were significantly lower in comparison with cryopreserved apices subjected to BAP during pre-treatment. In contrast, post-thaw recovery rates of shoot tips excised from plants exposed to BAP during long-term culture were in accord with the recovering ability of apices exposed to both long and short-term influence of BAP. The results indicate that long-term exposure of plant material to BAP may result in a delay of ABA protection effect, but the presence of BAP in the medium during the short-term pre-culture period was indispensable for improvement in ability to recover after cryopreservation.

Influence of BAP on shoot tip anatomy during short/long-term culture

The size of the apical meristem, its compactness and position of the first leaf primordia were examined by light microscopy. Comparison of shoot tips subjected to short and/or long-term cultivation in the presence of BAP revealed anatomical differences which may contribute to overall tolerance of cryogenic temperatures in explants. The microscopic observations indicated that shoot tip meristematic domes of the plants exposed to BAP during the pre-culture period only were nearly fully covered with the first pair of leaf primordia (Fig. 2A, C), which were noticeably bigger than those of the plants isolated from clusters formed under long-term influence of BAP. Enlarged leaf primordia may provide better protection of the meristematic dome against low temperatures during cryopreservation. On the other hand, shoot tips of cluster-derived plants differentiated during long-term exposure to BAP possessed meristematic domes of comparable size, but unprotected by the first pair of leaves (Fig. 2B, D). More intensive colouring of the central zone by safranin indicated higher cell division frequency in cluster-derived plants with more dilated intercellular spaces (Fig. 2D). In order to discover anatomical and morphological differences caused by predicted interaction between BAP and ABA during the pre-treatment period, we examined the influence of short-term exposure of shoot tips to BAP as well. To this end, the anatomy of shoot tips excised from plants cultured on the hormone-free medium and subsequently exposed to liquid pre-culture medium supplemented with both BAP and ABA for 10 days was compared with the same set of samples pre-cultured in the liquid medium enriched with ABA only. In comparison with the non-treated apices cultured on the basal medium (Fig. 2 A, C), both sets of pre-treated shoot tips possessed visibly bigger apical meristem regions (Fig. 3 C, D). In the case of explants pre-cultured in liquid medium supplemented with BAP and ABA, their meristematic centres were compact and fully covered by the first pair of leaf primordia (Fig. 3 A, C). In contrast, shoot tips pre-cultured solely in the liquid ABA-enriched medium possessed wholly uncovered central zones because of the only-developing leaf primordia (Fig. 3 B, D), despite similar meristematic dome size. Furthermore, larger intercellular spaces observed in these structures indicated decreased compactness of shoot apices (Fig. 3 D). The results show that long-term exposure of plants to BAP may delay the protective role of ABA during pre-culture, which led into reduced ability to withstand cryopreservation as a consequence of the uncovered meristematic zone. On the other hand, short-term influence of BAP induced anatomical and morphological alterations causing higher levels of meristem compactness and protection by leaf primordia, which resulted in increased tolerance of low temperatures.

Table 1. Recovery rates of cryopreserved *H. perforatum* shoot tips derived from plants growing on the basal RM medium (BAP-free) using 0.3M sucrose (A) and 0.076µM ABA (B) as preculture additives, each in three different time durations (16, 24, 48 hours; 3, 7, 10 days).

A	0.3M sucrose			**B**	0.076µM ABA		
†genotype	*16 hours* ^{y}	*24 hours* ^{xy}	*48 hours* ^{x}	†genotype	*3 days* ^{z}	*7 days* ^{xy}	*10 days* ^{x}
5/7/2^{ab}	0	3.3	11.8	**5/7/2**^{bc}	15	21	62
5/7/4^{bc}	16	7.1	16.1	**5/7/4**^{abc}	6.7	13.6	38.9
24/7/5^{ab}	3.6	4.2	8.3	**24/7/5**^{abc}	15	55	25.7
29/7/5^{c}	17.9	26.1	10.3	**29/7/5**^{c}	56.3	42	29.4
34/7/1^{d}	28.6	50	36	**34/7/1**^{abc}	10	3.7	38.7
36/7/2^{a}	0	0	0	**36/7/2**^{abc}	13.8	26.9	13.9
40/7/2^{a}	0	0	6.3	**40/7/2**^{ab}	10	3.7	23.5
40/7/3^{a}	0	0	6.7	**40/7/3**^{abc}	3.9	33.3	12.9
42/7/3^{ab}	0	0	12.5	**42/7/3**^{a}	7.4	6.3	5.7
42/7/5^{ab}	0	6.7	3.5	**42/7/5**^{ab}	4.4	9.5	15.4
Mean recovery rate (%)	6.6	8.6	12.4	Mean recovery rate (%)	12.3	22.5	24.6

Statistical differences between recovery rates after various pre-culture durations (x, y, z) and among genotypes (a, b, c) are marked by superscripts.†The triple numbers represent different *H. perforatum* genotypes, originated from one diploid mother plant. Common ancestor is expressed by the same number in the middle.

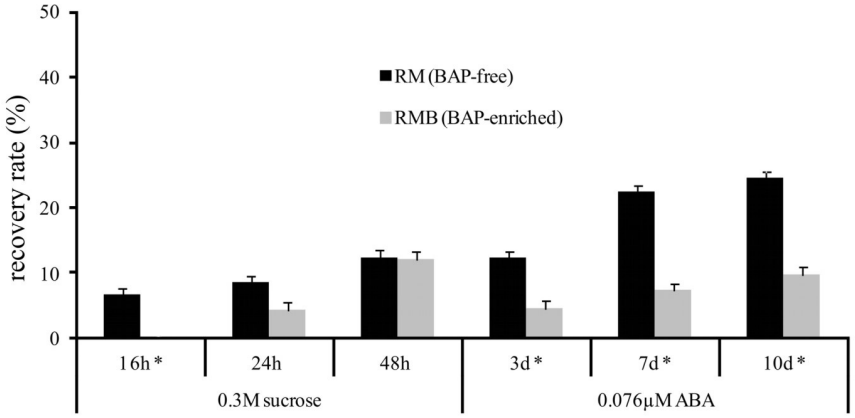

Fig 1. Comparison of average recovery rates (±SEM) of *H. perforatum* L. shoot tips cultured on BAP-free and BAP-supplemented medium, respectively analysed at P=0.05; statistically significant differences are marked by asterisks.

Discussion

Genetic pre-disposition, culture conditions prior to cryogenic treatment and anatomical differences could markedly affect the success of the cryopreservation procedure. The primary aim of this work was to study the effect of genotype on the tolerance of *H. perforatum* shoot tips to cryogenic temperatures in terms of post-cryogenic recovery, the interaction between cytokinin and ABA after long/short-term exposure to BAP, and the resultant anatomical and morphological alterations in the apical meristem. Experimental material included two sets of shoot tips excised from individual plants cultured on the hormone-free medium and from clusters formed as a result of long-term cultivation on BAP-supplemented medium respectively. These were further pre-treated for a short time with sucrose or ABA and cryopreserved by vitrification.

Despite their different action modes, sucrose and ABA are widely-used preculture agents contributing to the pre-conditioning of plant material before exposure to a cryogenic environment. The protective role of sucrose as a disaccharide able to penetrate cell walls lies in the maintenance of plasma membranes in liquid crystalline state. This sugar is also assumed to prevent protein denaturation because of its capability to form bonds with biomacromolecules (Hoekstra et al., 2001). Sucrose pre-culture has also been observed to have a contributing effect on the reduction of freezable water content in the cells (Panis et al., 1996). On the other hand, ABA as a phytohormone is involved in plant responses to many environmental abiotic stresses including cold. Furthermore, acting as a signal molecule, ABA triggers the expression of gene encoding for enzymes protecting the plant and preserving membranes against low temperature injuries (Minami et al., 2003; Pearce, 2004) or toxicity of cryoprotectants (Seki et al., 2003).

Genotype-specific response to cryogenic treatment

In this study, we recorded different capabilities of individual genotypes to withstand cryogenic temperature, despite all genotypes represented the seed progenies of one diploid mother plant. The genetic background of this variation might arise from a high degree of heterozygosity in diploids, which

Table 2. Recovery percentages of cryopreserved shoot apices derived from *H. perforatum* clusters originated on the BAP-enriched medium using 0.3M sucrose (A) and 0.076µM ABA (B) as preculture agents, each in three different durations (16, 24, 48 hours; 3, 7, 10 days).

A	0.3M sucrose			**B**	0.076µM ABA		
†genotype	*16 hours*	*24 hours* ^{y}	*48 hours* ^{x}	†genotype	*3 days* ^{z}	*7 days* ^{xy}	*10 days* ^{x}
5/7/2^{ab}	0	0	20	**5/7/2**^{ab}	3.3	15.4	16.2
5/7/4^{ab}	0	2.9	13.3	**5/7/4**^{ab}	16	3.9	2.9
24/7/5^{a}	0	6.5	0	**24/7/5**^{b}	37.5	3.3	21
29/7/5^{ab}	0	8.7	19.2	**29/7/5**^{ab}	6.9	13	15.4
34/7/1^{ab}	0	3.5	16.7	**34/7/1**^{ab}	6.1	0	14.3
36/7/2^{a}	0	0	2.9	**36/7/2**^{ab}	0	25	3.2
40/7/2^{a}	0	0	3.1	**40/7/2**^{ab}	10.5	8.3	9.4
40/7/3^{b}	0	17.6	40	**40/7/3**^{a}	7.3	4.2	0
42/7/3^{ab}	0	3.1	10.3	**42/7/3**^{ab}	15.6	3.9	0
42/7/5^{a}	0	6.5	3.7	**42/7/5**^{ab}	5.9	3.7	10.8
Mean recovery rate (**%**)	0	4.4	12.2	Mean recovery rate (**%**)	4.6	7.3	9.8

Statistical differences between post-cryogenic recovery rates after different pre-culture durations (x, y, z) and inter-genotype variances (a, b, c) are marked by superscripts. †The triple numbers represent different *H. perforatum* genotypes, originated from one diploid mother plant. Common ancestor is expressed by the same number in the middle.

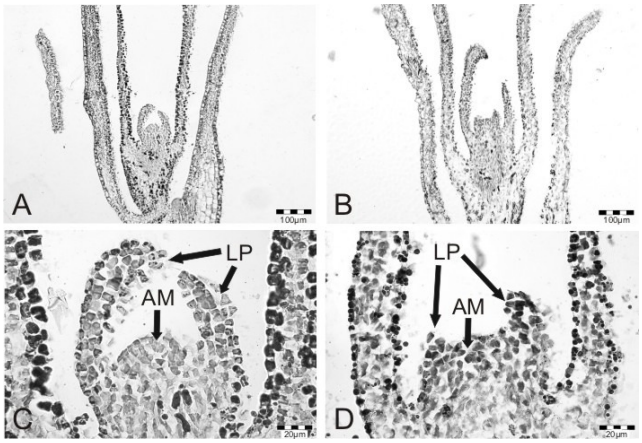

Fig 2. Longitudinal sections of *H. perforatum* L. shoot apices before cryopreservation procedure observed by light microscopy. Tips isolated from basal RM medium cultivated plants (A and C) possessed their meristematic domes protected by leaf primordia, whereas meristems of apices isolated from BAP-enriched medium cultivated clusters were uncovered (B and D). LP = leaf primordium, AM = apical meristem.

were found to reproduce mostly and obligatorily sexually (Koperdáková et al., 2004). Similar results documenting a broad variation range of recovery rates among genotypes were obtained by Sales et al. (2001) for *Digitalis obscura* L. and by Kaity et al. (2008) for *Carica papaya* L. shoot tips. However, the influence of genotype was statistically significant solely in the case of shoot tips excised from plants cultured on the hormone-free medium.

Response of shoot tips to different pre-culture agents

Our results reveal the dependence of average post-cryopreservation recovery rates on the pre-treatment duration. Effective sucrose pre-culture time may vary among plant species, e.g. *Rauvolfia serpentina* L. explants reached their maximum recovery rates after 4 days of sucrose exposure (Ray and Bhattacharya, 2008), whereas *Fragaria ananassa* Duch. shoot tips did so after 24 hour long pre-treatment (Pinker et al., 2009). *H. perforatum* shoot tips reached the highest average capability to recover after 48 hour long exposure to sucrose. However, several genotypes showed notably improved ability to recover even after shorter pre-culture periods. Subjection of explants to ABA during pre-culture markedly enhanced their average recovery rates, which is in accord with the results of Lu et al. (2009), who recorded increased survival abilities of *Ginkgo biloba* L. cell cultures after ABA preculture in comparison with sucrose treatment. However, average recovery rates were relatively low (<50%) originating from both genotype-specific contribution and possible antagonistic effect between glycerol and ABA during loading and cryoprotective treatment, as recorded by Mallón et al. (2008). Furthermore, recovery rates of *H. perforatum* shoot tips also showed a tendency to rise with pre-culture duration, with the highest average recoveries achieved after 10 day long pre-treatment. The effectiveness and rate of ABA action may vary among plant species, which is supported by the findings of several authors (Burritt, 2008; Na and Kondo, 1996), showing

Table 3. Recovery rates of cryopreserved shoot tips isolated from plants cultivated on the basal medium (hormone-free) and from clusters cultured on the BAP-enriched medium. Data are showing recovery percentages after cryopreservation with modified pre-treatment (liquid medium supplemented with ABA only).

	Long-term culture	
†genotype	hormone-free	BAP-enriched
5/7/2	6.3	7.4
5/7/4	3.5	3.4
24/7/5	10.7	13.6
29/7/5	3.5	0
34/7/1	13.8	12.9
36/7/2	6.7	5.9
40/7/2	0	3.2
43/7/3	6.5	11.5
42/7/3	3.3	16.7
42/7/5	0	14.8
Mean recovery rate (%)	5.4	8.9

The triple numbers represent different *H. perforatum* genotypes, originated from one diploid mother plant. Common ancestor is expressed by the same number in the middle.

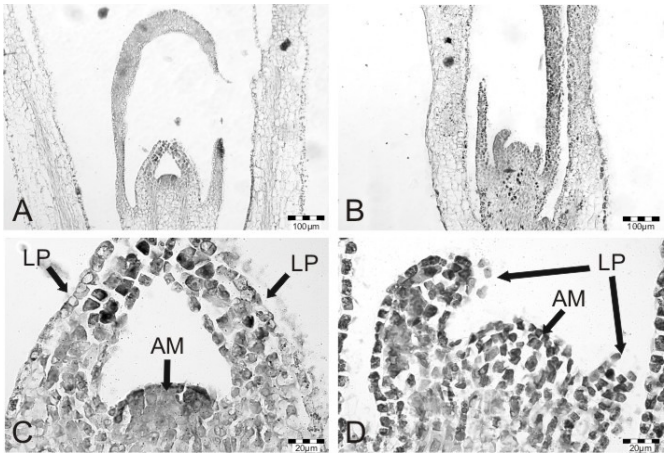

Fig 3. Longitudinal sections of *H. perforatum* L. shoot tips after preculture observed by light microscopy. Shoot apices isolated from individually growing plants (basal RM medium) after 10 days long RMB+ABA pre-treatment (A and C) were observed to have their meristematic domes covered by leaf primordia. Apical meristems of tips precultured for 10 days with RM+ABA medium (B and D) reported the increased sizes with conspicuous intercellular spaces. LP = leaf primordium, AM = apical meristem

maximum tolerance of cryogenic temperatures after short-term ABA pre-culture.

Interaction between ABA and cytokinins

Despite both sets of plant material being subjected to the same cryopreservation procedure, statistical assessments revealed significant differences in average recoveries between BAP-enriched and hormone-free long-term culture conditions, apparent especially after pre-treatment with ABA. The shoot tips isolated from *H. perforatum* plants exposed to BAP during long-term pre-culture showed impaired capability to withstand cryogenic temperatures, presumably as a consequence of delayed ABA action during pre-treatment. This synthetic cytokinin is known to induce multiple shoot formation in tissue cultures through apical dominance suppression, which triggers cluster differentiation. Despite its role being quite well documented, the exact mechanism of its action is still unclear. Although this cytokinin does not naturally occur in plants, BAP successfully regulates cell division, stimulates adventitious shoot initiation and inhibits root formation, and thus becomes very useful for gaining sufficient amounts of plant material during a short time period (Taiz and Zeiger, 2003). This cytokinin also functions as an efficient multiple shoot formation agent for *H. perforatum* explants (Čellárová et al., 1992). The antagonistic effects between cytokinins and ABA are known in higher plants, especially under physiological conditions (Werner and Schmülling, 2009). However, it is known that BAP may reduce the ability of plants to acclimatize to cold due to its predicted interaction with ABA during hardening (Baldwin et al., 1998). Moreover, Reed (1993) noticed decreased tolerance of *Rubus* sp. shoot tips induced by BAP, which was partly genotype-dependent. Based on our results, we suggest that the interference between BAP and ABA could represent a presumable reason for decreased recovery rates. To discover whether the predicted interference between ABA and cytokinin occurs as a consequence of long or short-term exposure to BAP, both sets of *H. perforatum* shoot tips were cryopreserved after ABA pre-treatment without BAP in the pre-culture medium. Interestingly, the results show the lowest recoveries of both sets. Similarly, pre-culture of plant material in BAP-free medium supplemented with ABA prevented the post-thaw ability to recover in *Gentiana scabra* Bunge shoot tips (Suzuki et al., 2006). These results suggest that short-term exposure of explants to BAP is beneficial for improved recovery rate of *H. perforatum* shoot apices. In contrast, long-term subjection to BAP delayed the effect of ABA and thus reduced the ability to recover after cryopreservation.

Diverse low temperature tolerance rates were found not only at the level of plant recovery, but several dissimilarities were also observed in the anatomy of shoot tips. Whereas longitudinal sections of the apices isolated from *H. perforatum* plants growing on the hormone-free medium possessed central zones covered by the first pair of leaf primordial, the shoot tips derived from clusters differentiated after long-term exposure to BAP were much smaller and uncovered. These observations are in accord with Helliot et al. (2002), who found that actively-proliferating meristems of *Mus*a sp. were not protected by leaf primordia. Furthermore, the size of the apical meristem was comparable in both sets, which could be caused by redistributed impact of exogenous cytokinin on the individual shoots of the cluster.

Effect of exposure duration to BAP on shoot tip anatomy

Anatomical study of shoot tips isolated from plants growing on the hormone-free medium and pre-cultured with ABA in the presence or absence of BAP revealed markedly enlarged apical meristems. However, the apical meristem regions of shoot tips exposed to BAP for a short time were intensively stained by safranin, which indicated their compactness and increased mitotic activity. On the other hand, the shoot apices pre-cultured in BAP-free medium possessed markedly larger intercellular spaces, indicating decreased meristem compactness. The disintegration of meristematic tissue probably led to the significantly decreased recovery rates. Similar results were recorded by Thinh (1997), who clearly

demonstrated that the physiological status of plant material plays an important role in the post-thaw recovery phase. In the case of shoot tips, the apical dome should be at least partially covered and thus protected by the first pair of leaf primordia, which corresponds with our microscopic observations. Despite these facts, the exact mechanism of interaction between ABA and BAP remains unresolved.

The present study reveals substantial differences in the response of individual *H. perforatum* genotypes originated from one mother plant to cryogenic treatment. Post-thaw recovery rates appeared to be dependent on the pre-culture additive and pre-treatment duration. Besides genetic background and pre-cryogenic treatment, growth regulators such as cytokinins added into the culture medium could counteract the effect of the pre-culture additive. Moreover, the interaction between BAP and ABA resulted in anatomical alterations of shoot apices which contributed to the decreased post-cryogenic recovery rate. In conclusion, the establishment of an effective cryopreservation protocol for *H. perforatum* should include not only modifications of particular external parameters, but rather more profound study of the physiological status of plants prior to cryopreservation and their genetic background.

Materials and methods

Plant material and culture conditions

Plant material used in the experiments included *in vitro* grown seed-derived plants of 10 diploid (2n = 2x = 16) *Hypericum perforatum* L. progenies originated from one diploid mother plant. The seeds were surface-sterilised with 1% (w/v) $AgNO_3$ for 15 min and washed 4-5 times in sterile distilled water. Subsequently they were placed on the surface of basal RM medium containing Linsmaier-Skoog`s salt mixture (Linsmaier and Skoog, 1965), Gamborg`s B5 vitamins (Gamborg et al., 1968), 30 $g.l^{-1}$ sucrose, 2 $mg.l^{-1}$ glycine and 100 $mg.l^{-1}$ myo-inositol with pH adjusted to 5.6 before autoclaving. Seedlings were cultured at 23 °C temperature and 40% relative humidity, under 16 h /8 h photoperiod and artificial irradiance of 30 $\mu E.m^{-2}.s^{-1}$. After 3-4 weeks of cultivation, shoot tips with two leaf primordia were excised and transferred either onto basal medium RM or the same medium supplemented with 0.5 $mg.l^{-1}$ benzylaminopurine (BAP; RMB medium).

Cryopreservation procedure

Shoot tips were isolated from plants cultivated on the RM medium or on the RMB, then transferred to the liquid RMB medium (pH = 5.6) supplemented with either 0.3M sucrose or 0.076µM abscisic acid (ABA) or to the liquid RM medium (pH = 5.6) containing ABA. Pre-culture on sucrose lasted 16, 24 or 48 hours and ABA-preculture 3, 7 or 10 days, both on the orbitary shaker at 120 rpm. After pre-treatment with either ABA or sucrose, shoot tips were treated for 20 min with loading solution (LS, pH = 5.6) composed of liquid RM medium supplemented with 2M glycerol and 0.4M sucrose on the orbitary shaker under laboratory temperature. Following LS treatment explants were transferred into sterile cryotubes (2 ml, Sarstedt) containing 1 ml of plant vitrification solution (PVS3) consisting of 50 % (w/v) glycerol and 50 % (w/v) sucrose (Nishizawa et al., 1993). After 90 minutes of equilibration on ice the samples were immersed directly into liquid nitrogen (LN, -196 °C) and stored for at least 24 hours. Thawing was performed in a water bath at 40 °C for 1 min and then the explants were washed for 20 min in the liquid RM medium supplemented with 1.2M sucrose on the orbitary shaker. Thawed shoot tips were placed onto RM medium moistened filter followed by transfer onto solid RMB medium. Subsequently the regeneration phase took place in darkness for 14 days, followed by exposure to light conditions as stated above. The recovery rate was defined as the percentage of shoot tips able to regrow.

Statistical evaluation

Statistical evaluation of experimental results was conducted using the STATGRAPHICS version 5.0 package. The experiments were performed using pseudo replication design in three replicates. Each treatment was carried out with overall 30 shoot tips (10 per replicate). Average recovery rates for each pre-culture agent were compared using Mann-Whitney U–test, and the effect of genotype on the ability to recover was assessed using one-way ANOVA ($P \leq 0.05$).

Preparation of histological slides

Five shoot tips were excised from both types of experimental plants (grown on the RM or RMB medium) and from those pre-treated with liquid RM/RMB medium supplemented with 0.076µM ABA. The apices were fixed using freshly-prepared solution consisting of 4.0% paraformaldehyde, 0.25% glutaraldehyde and 0.1M sodium phosphate buffer (ph = 7.0) for 30 min in vacuum and 3 hours on ice. Fixed tips were subsequently dehydrated in various aqueous ethanol solutions, infiltrated by ethanol - xylene mixtures in different v/v ratios, penetrated with xylene – paraplast combinations and finally incubated in 100% paraplast in accordance with Schmeltzer et al. (1989). Hardened paraplast blocks were sectioned at 7µm thickness with a standard rotary microtome and deparaffinised by imbibition in 100% xylene, decreasing concentrations of ethanol (100%, 96%, 70%, 50% and 30%) and in distilled water. The slices were stained using Safranin-Fast green procedure and observed under light microscope (Olympus BX 51, Japan).

Acknowledgements

The authors wish to thank Dr. Martina Urbanová for her contribution to the experimental design, Zdenka Lacková for preparation of histological slides and Andrew Billingham for the linguistic review of the paper. This work was supported by the Slovak Research and Development Agency under contract SRDA 0040-10, the project Network of Excellence in Oncology (NEXO) No 26220120039 funded from EU structural funds, the Scientific Grant Agency of the Slovak Ministry of Education No 1/142/11.

References

Baldwin BD, Bandara MS, Tanino KK (1998) Is tissue culture a viable system with which to examine environmental and hormonal regulation of cold acclimation in woody plants? Physiol Plantarum. 102:201-209

Burritt DJ (2008) Efficient cryopreservation of adventitious shoots of Begonia x erythrophylla using encapsulation-dehydration requires pretreatment with both ABA and proline. Plant Cell Tiss Organ Cult. 95:209-215

Čellárová E, Kimáková K, Brutovská R (1992) Multiple shoot formation and phenotypic changes of R_0 regenerants in *Hypericum perforatum* L. Acta Biotechnol. 12:445-452

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res. 50:151-158

Havlová M, Dobrev PI, Motyka V, Štorchová H, Libus J, Dobrá J, Malbeck J, Gaudinová A, Vanková R (2008) The role of cytokinins in responses to water deficit in tobacco plants over-expressing *trans*-zeatin O-glucosyltransferase gene under *35S* or *SAG12* promoters. Plant Cell Environ. 31:341-353

Helliot B, Panis B, Poumay Y, Swennen R, Lepoivre P, Frison E (2002) Cryopreservation for the elimination of cucumber mosaic and banana streak virus from banana (*Musa* spp.). Plant Cell Rep. 20:1117-1122

Hoekstra FA, Golovina EA, Tetteroo FAA, Wolkers WF (2001) Induction of desiccation tolerance in plant somatic embryos: How exclusive is the protective role of sugars? Cryobiology. 43:140-150

Kaity A, Ashmore SE, Drew RA, Dulloo ME (2008) Assessment of genetic and epigenetic changes following cryopreservation in papaya. Plant Cell Rep. 27:1529-1539

Koperdáková J, Brutovská R, Čellárová E (2004) Reproduction pathway analysis of several *Hypericum perforatum* L. somaclonal families. Hereditas. 140:34-41

Linsmaier EM, Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. Plant Physiol. 18:100-127

Lu ZW, Popova EV, Wu ChH, Lee EJ, Hahn EJ, Paek KY (2009) Cryopreservation of *Ginkgo biloba* cell culture: Effect of pretreatment with sucrose and ABA. Cryo Lett. 30:232-243

Mallón R, Bunn E, Turner SR, González ML (2008) Cryopreservation of Centaurea ultreiae (Compositae) A critically endangered species from Galicia (Spain). Cryo Lett. 29:363-370

Minami A, Nagao M, Arakawa K, Fujikawa S, Takezawa D (2003) Abscisic acid-induced freezing tolerance in the moss *Physcomitrella patens* is accompanied by increased expression of stress-related genes. J Plant Physiol. 160:475-483

Na HY, Kondo K (1996) Cryopreservation of tissue-cultured shoot primordial from shoot apices of cultured protocorms in *Vanda pumila* following ABA preculture and desiccation. Plant Sci. 118:195-201

Nishizawa S, Sakai A, Amano Y (1993) Cryopreservation of asparagus (*Asparagus officinalis* L.) embryogenic suspension cells and subsequent plant rageneration by vitrification. Plant Sci. 91:67-73

Panis B, Totte N, Van Nimmen K, Withers LA, Swennen R (1996) Cryopreservation of banana (*Musa* spp) meristem cultures after preculture on sucrose. Plant Sci. 121:95-106

Pearce DW, Rood SB, Wu RL (2004) Phytohormones and shoot growth in a three-generation hybrid poplar family. Tree Physiol. 24:217-224

Pence VC (1999) The application of biotechnology for the conservation of endangered plants. In: Benson EE (ed) Plant Conservation Biotechnology, Taylor & Francis, London

Pinker I, Halmagyi A, Olbricht K (2009) Effect of sucrose preculture on cryopreservation by droplet-vitrification *of strawberry* cultivars and morphological stability of cryopreserved plants. Cryo Lett. 30:202-211

Ray A, Bhattacharya S (2008) Cryopreservation of *in vitro* grown nodal segments of *Rauvolfia serpentina* by PVS2 vitrification. Cryo Lett. 29:321-328

Reed BM (1993) Responses to ABA and cold-acclimation are genotype dependent for cryopreserved blackberry and raspberry meristems. Cryobiology. 30:179-184

Sales E, Nebauer SG, Arrillaga I, Segura J (2001) Cryopreservation of *Digitalis obscura* selected genotypes by encapsulation-dehydration. Planta Med. 67:833-838

Schmeltzer E, Krüger-Lebus S, Hahlbrock K (1989) Temporal and spatial patterns of gene expression around sites of attempted funga1 infection in parsley leaves. Plant Cell. 1:993-1001

Seki M, Kamei A, Yamaguchi-Shinozaki K, Shinozaki K (2003) Molecular responses to drought, salinity and frost: common and different paths for plant protection. Curr Opin Biotech. 14:194-199

Skyba M, Urbanová M, Kapchina-Toteva V, Košuth J, Harding K, Čellárová E (2010) Physiological, biochemical and molecular characteristics of cryopreserved *Hypericum perforatum* L. shoot tips. Cryo Lett. 31:249-260

Suzuki M, Ishikawa M, Okuda H, Noda K, Kishimoto T, Nakamura T, Ogiwara I, Shimura I, Akihama T (2006) Physiological changes in Gentian axillary buds during two-step preculturing with sucrose that conferred high levels of tolerance to desiccation and cryopreservation. Ann Bot – London. 97:1073-1081

Taiz L, Zeiger E (2003) Plant Physiology. 3rd edn, Sinauer Assoc Inc Publ, Sunderland

Thinh NT (1997) Cryopreservation of germplasm of vegetatively propagated tropical monocots by vitrification. Doctoral Paper, Faculty of Agriculture, Kobe University, Japan

Tran L–SP, Urao T, Qin F, Maruyama K, Kakimoto T, Shinozaki K, Yamaguchi–Shinozaki K (2007) Functional analysis of AHK1/ATHK1 and cytokinin receptor histidine kinases in response to abscisic acid, drought, and salt stress in *Arabidopsis*. PNAS. 104:20623-20628

Werner T, Schmülling T (2009) Cytokinin action in plant development. Curr Opin Biol. 12:527-538

Yoshimatsu K, Touno K, Shimomura K (2000) Cryopreservation of medicinal plant resources: retention of biosynthetic capabilities in transformed cultures. In: Engelmann F, Takagi H (eds) Cryopreservation of Tropical Plant Germplasm: Current Research Progress and Application. International Agriculture Series 8. JIRCAS, Rome, pp 77–88