

Impact of pre-culture on short- and long-term in vitro recovery of the biosynthetic potential and enzymatic and non-enzymatic antioxidant defense of *Hypericum rumeliacum* Boiss. after cryostorage

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Abstract Due to its high hypericin and pseudohypericin in vitro biosynthetic capacity, the Balkan endemic *Hypericum rumeliacum* was selected as a prospective candidate for long-term preservation of valuable medicinal plant germplasm. Initial cryopreservation experiments were previously conducted based on the successful protocol established and reported for the widely studied *H. perforatum*. This is the first report on the impact of pre-culture duration on the short- and long-term in vitro recovery of the biosynthetic potential and antioxidant defense system of *H. rumeliacum* cryopreserved by vitrification. Cryopreservation did not impair the phenolics and flavonoids production of the regenerated plants. Moreover, hypericin and pseudohypericin levels even increased substantially in one of the regenerated lines, reaching yields from 0.107 and 0.752 mg g⁻¹ DW in the control up to 0.277 and 1.112 mg g⁻¹ DW for hypericin and pseudohypericin, respectively. However, the physical injury stress of the pre-culture treatment manipulations affected the physiological status of regenerants in a time dependent manner. Within 6 months after thawing, regenerants with the highest oxidative stress after pre-culture, were characterized with an augmentation of antioxidant metabolites such as phenolics, flavonoids, glutathione and ascorbic acid as well as

increased antioxidant enzymatic activities in comparison with both the non-frozen control and the regenerants with the lowest pre-culture oxidative stress. Then, after 18 months of recovery, the same first *H. rumeliacum* group displayed a marked drop of enzymatic antioxidant activity as compared with the other groups of plants. Further research is needed to target oxidative stress alleviation to optimize *H. rumeliacum* cryopreservation protocol.

Keywords Antioxidant enzymes · Cryogenic storage · Hypericins · *Hypericum rumeliacum* · Polyphenolics

Abbreviations

ABA	Abscisic acid
APX	Ascorbate peroxidase
BA	Benzyl adenine
LS	Loading solution
PVS	Plant vitrifying solution
MDA	Malondialdehyde
H ₂ O ₂	Hydrogen peroxide
FW	Fresh weight
DW	Dry weight
DMFA	Dimethylformamide
PVP	Polyvinylpyrrolidone
EDTA	Ethylenediaminetetraacetic acid
PAL	Phenylalanine ammonia-lyase
CAT	Catalase
GR	Glutathione reductase
GSSG	Oxidised glutathione
GSH	Reduced glutathione
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
AsA	Ascorbic acid
DHA	Dehydroascorbate

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Introduction

The Balkan endemic *Hypericum rumeliacum* Boiss. (Guttiferae) belongs to the *Drosocarpium* Spach. section of the *Olympia* group of the genus. Recent studies report on its phytochemical composition and valuable pharmacological properties (Galati et al. 2008). Infrageneric chemotaxonomic surveys provide suggestions on hypericins-rich *Hypericum* species, assuming that such species as *H. boissieri*, *H. barbatum* and *H. rumeliacum* of this section might contain two to fourfold higher amounts of hypericins than *H. perforatum* (Bruni and Sacchetti 2009). Nevertheless, despite the intensive biotechnological research of secondary metabolites production in *Hypericum* (Kirakosyan et al. 2004), the *Drosocarpium* section has been neglected in this aspect so far. Our previous research showed that shoot cultures of *H. rumeliacum* produce high phenolic and flavonoid levels, commensurable to the intact plant. An effective in vitro multiplication system with preservation of the phenolic biosynthetic capacity of the species was developed (Danova et al. 2010). Vitamin modification of the medium afforded stimulation of hypericin and pseudohypericin production, exceeding the values for the other *Hypericum* species studied by us, or reported in literature (excluding elicitation models). However, the inherent high hypericins production seemed to be connected to a significant augmentation of oxidative stress compared to the other studied low- or hypericin non-producing *Hypericum* species (Danova et al. 2012). Due to its high biosynthetic potential, *H. rumeliacum* was selected for the further development of approaches for its long-term preservation as a biotechnological producer of pharmacologically relevant secondary metabolites.

Cryopreservation is a prospective method for the long-term conservation of plant germplasm, as the initial traits of the material are most often preserved. The storage at ultra-low temperatures (-196°C) has a practical application for the preservation of plant cells and tissues, characterized by high production of valuable secondary metabolites. A number of cryopreservation techniques have been successfully applied to different in vitro cultures: cell suspensions, callus, shoots, somatic and zygotic embryos of medicinal plants (Bajaj 1995; Engelmann 1997; Panis and Lambardi 2005; Benson 2008). Throughout the years, fundamental research as well as empirical experience has led to the development of a number of protocols, technologies and instrumentation for the preservation of plant germplasm (Benson 2008). Intra-cellular ice crystal formation during freezing and consequent membrane damage are a critical point in cryopreservation and therefore bring out optimal dehydration of the living plant tissue as a core question to be solved. Depending on the freezing method, different approaches have been extensively studied and

developed: (i) either controlled, slow cooling to about (-80°C) in which the gradual temperature drop causes an extra-cellular ice formation and subsequent osmotic “freeze-dehydration” of the cell, followed then by liquid nitrogen immersion or (ii) vitrification—dehydration is achieved osmotically through the application of diverse cryoprotectant solutions; then tissues are directly immersed into liquid nitrogen (-196°C) resulting in a rapid phase transition from liquid to amorphous state, avoiding ice crystal formation. Hence, the great diversity of approaches for dehydration and cryoprotection of the plant tissues makes cryopreservation a complex and multi-step process (Engelmann 1997; Panis and Lambardi 2005; Benson 2008 and references cited therein). A crucial step in the cryopreservation protocol is the induction of tolerance in the plant tissue or organ, in order to survive the extreme dehydration before immersion in liquid nitrogen (Suzuki et al. 2006). This is usually achieved by pre-culturing with sucrose, abscisic acid or cold-hardening (Suzuki et al. 2006 and references cited within). Adaptive metabolism (hardening) aims at inducing the ability of the plant to survive unfavorable environmental stress. One of the approaches for accomplishing this effect is the exogenous application of abscisic acid (Panis and Lambardi 2005). Abscisic acid has been shown to play an important role in plant water balance and in the adaptation of plants to environmental stress. Moreover, pre-culture of cell suspensions or tissues with ABA has also shown in some cases to enhance freezing or desiccation tolerance, both properties desirable for cryopreservation (Shimonishi et al. 1991; Kahn et al. 1993; Gusta et al. 1996).

Successful cryopreservation, based on ABA pre-culture, was reported for *H. perforatum* resulting in preservation of its genetic stability, as well as the total hypericins levels (Urbanová et al. 2002, 2006). Based on this protocol, initial experiments on cryopreservation of *H. rumeliacum* by vitrification after $0.076\text{ }\mu\text{M}$ abscisic acid (ABA) pre-culture were previously performed (Danova et al. 2009a, b). The obtained low survival rates after cryostorage (1.4, 2.2 and 1.3 % after 3, 7 and 10 days of pre-culture, respectively) motivated us to carry out a broader research on the physiological and biochemical parameters of the plants during pre-culture and after thawing and attempt to interpret the possible relations between them. The physiological status of *H. rumeliacum* apical explants during the pre-culture step was investigated. It was established that by prolonging its duration, a decrease of oxidative stress was achieved in the explants which were to further enter dehydration and liquid nitrogen immersion (Danova 2010). Thus, explants pretreated for 3 days were characterized with the highest levels of oxidative stress markers, while after 10 days ABA pre-culture, these parameters were the lowest. It has to be noted, that the same effect was observed

in *H. rumeliacum* explants in both ABA-supplemented and control (ABA-lacking) pre-culture media.

Therefore the aims of the present study were: (i) to assess the impact of pre-culture duration on the biosynthetic capacity of regenerated plants; (ii) to assess the status of the enzymatic and non-enzymatic antioxidant defense of the short- and long-term recovered plants; (iii) to study the possible correlations between the physiological status of explants after the pre-culture step and the status of the short- and long-term regenerated plants.

Materials and methods

Plant material and culture conditions

Intact plant material of *Hypericum rumeliacum* Boiss. was collected at its natural habitat in Bulgaria. Voucher specimen was deposited at the Herbarium of the Institute for Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences (SOM 163 524). In vitro shoot cultures were induced from surface-sterilized mono-nodal stem segments of the in situ growing wild plants. Shoots were further maintained on solidified Murashige and Skoog (1962) culture medium at 25 °C, 16/8 h photoperiod and irradiation intensity of 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$, with a 45 days period of regular subculture (Danova et al. 2010).

Dry weight determination

For the FW/DW ratio determination, fresh plant material was distributed into four portions of 0.3 g each and dried at 105 °C for 5 h. The FW/DW was calculated for each individual portion and the average value presented. For the purpose of secondary metabolites determination, samples were dried at 40 °C until constant weight.

Cryopreservation of *Hypericum rumeliacum* shoot tip meristems

Hypericum rumeliacum apical shoot meristems with two primordial leaves were excised from the in vitro cultured shoots of the plant. Further, pre-culture consisted of 0.076 μM ABA treatment of the explants in a liquid Murashige and Skoog's (1962) medium supplemented with Gamborg et al. (1968) vitamins, 100 mg l^{-1} myo-inositol, 30 g l^{-1} sucrose and 0.5 mg l^{-1} benzyl adenine (BA) (RMB_{0.5} medium). Three pre-culture periods had been experimented—3, 7 and 10 days (hereafter referred to as 3 days ABA, 7 days ABA and 10 days ABA, respectively). Then, the explants were immersed for 20 min in a loading solution containing 2 M glycerol and 0.4 M sucrose at room temperature. These shoot tips were dehydrated in

Plant Vitrifying Solution 3 (PVS3; Nishizawa et al. 1993) (50 % w/v sucrose and 50 % w/v glycerol) for 90 min on ice and finally directly immersed into liquid nitrogen (−196 °C). After 1 week of storage, thawing was performed in water bath at 40 °C for 1 min. Tips were rinsed in liquid RMB_{0.5} containing 1.2 M sucrose (unloading solution). Afterwards shoot tips were cultivated on semi-solid RMB_{0.5} for regeneration (Danova et al. 2009a, b). Survival was estimated as the percent of meristems capable of differentiating into plantlets. The regenerated plants were further cultivated in plant growth regulators-free medium, subcultured in every 45 days and subjected to biochemical investigations in the present work.

Total phenolics and flavonoids determination

100 mg DW of *H. rumeliacum* shoots, were extracted with hot 96 % (v/v) ethanol and then centrifuged at 15,000 rpm for 15 min. Total phenolics were determined by the Folin and Ciocalteu's colorimetric method of Singleton et al. (1999), modified by us as follows: an aliquot of the extract was placed in test-tube and distilled water, 1:1 Folin and Ciocalteu's reagent and 20 % Na_2CO_3 were added. The absorbance was measured at 730 nm and the total phenolics were calculated by means of a calibration curve of chlorogenic acid (in the range of 10–100 $\mu\text{g ml}^{-1}$) and expressed as mg of chlorogenic acid equivalent per gram DW of the sample. Total flavonoids content of the whole shoot samples was measured using a colorimetric assay in accordance with the method of Zhishen et al. (1999) modified as follows: 100 mg DW of the samples were extracted with hot 96 % (v/v) ethanol and then centrifuged at 15,000 rpm for 15 min. Aliquots of the extract were placed in test-tube and distilled water, 60 μl 5 % NaNO_2 and 60 μl 10 % AlCl_3 were added. After the addition of 1 N NaOH and distilled water, the absorbance at 510 nm was measured and the concentration was calculated by means of a calibration curve of (+)-catechin (in the range of 2–80 $\mu\text{g ml}^{-1}$). The total flavonoids of the samples were expressed in mg of (+)-catechin equivalent per gram DW of the sample. All measurements were performed in triplicate with three repetitions. Phenolic and flavonoid levels were studied for 3, and 10 days ABA and non-frozen controls, 6 months after thawing, and for 3, 7 and 10 days ABA and controls, 18 months after thawing.

Quantification of hypericin and pseudohypericin

For the estimation, 100 mg DW the whole shoots of *H. rumeliacum* were macerated for 30 min at room temperature with chloroform (anhydrous, $\geq 99\%$) and extracted in an ultrasonic bath (SIEL—UST1.6-100, Bulgaria) for 20 min. After filtration of the chloroform phase, the

material was covered with fresh chloroform and left for 48 h at room temperature. Then the ultrasonic extraction was repeated, the chloroform extract was discarded and the defatted plant material was repeatedly extracted with fresh portions (30 ml) of methanol in an ultrasonic bath until discoloration of the solvent. The combined methanolic extracts were evaporated in vacuum at 40 °C, filtered and adjusted to a volume of 10 ml in a volumetric flask. Hypericin (Hyp) and pseudohypericin (psHyp) were determined by a method for the RP HPLC simultaneous determination of hypericin and pseudohypericin as previously described (Danova et al. 2012): an Agilent 1100 series HPLC quaternary pump (Agilent Technologies, Inc. USA) equipped with multiple wavelength detector, micro vacuum degasser and a Rheodyne injector with 50 µl sample loop and a 250 mm × 4.6 mm Inertsil ODS2 column were used. A stepwise ternary gradient was employed using ethyl acetate (solvent A), methanol (solvent B) and 0.05 mol/l aqueous K₂CO₃ adjusted to pH 4 with formic acid (solvent C) as follows: from 10 % A/75 % B/15 % C to 20 % A/65 % B/15 % C over 15 min, changed to 50 % A/50 % B for 1 min and kept at this composition over 30 min. The flow rate was 1 ml/min and the sample size – 50 µl. A calibration graph was constructed using methanolic solutions of a Hypericin standard (95 %, HPLC grade, Fluka) in the interval of 0.006–0.095 mg ml⁻¹ Hypericin under exactly the same chromatographic conditions. The absorption was measured at 590 nm. All measurements were performed in triplicate. The detection limit of hypericin was 1 ng. Hypericins were measured for 3, 7 and 10 days ABA and controls, 18 months after thawing.

MDA and H₂O₂ determination

120 mg FW of the shoots were homogenized in a mortar at 4 °C with 0.1 % trichloroacetic acid and centrifuged for 20 min at 15,000 rpm. For malondialdehyde (MDA) estimation, 0.5 ml of the supernatant was mixed with 0.5 ml phosphate buffer pH 7.4 and 1 ml 0.5 % thiobarbituric acid dissolved in 20 % trichloroacetic acid and the samples were boiled for 30 min (Dhindsa et al. 1981). After rapid cooling of the samples in an ice-bath, absorption was measured at 532 and 600 nm using the extinction coefficient 155 mM⁻¹ cm⁻¹ (Heath and Packer 1968).

For the hydrogen peroxide (H₂O₂) assay, 0.5 ml of the supernatant was mixed with 0.5 ml phosphate buffer pH 7.4 and after the addition of 1 ml of 1 M KI, samples were incubated in the dark for 60 min and absorption was measured at 390 nm. The content was calculated using a standard curve of H₂O₂ in the range of 1–100 nmol ml⁻¹ of hydrogen peroxide (Jessup et al. 1994). MDA and H₂O₂ were studied for 3, and 10 days ABA and non-frozen

controls, 6 months after thawing, and for 3, 7 and 10 days ABA and controls, 18 months after thawing.

Photosynthetic pigments assay

Hypericum rumeliacum shoots were cut into 0.5 cm segments and mixed. 100 mg FW samples of the material were immersed into 5.0 ml DMFA (*dimethylformamide*) at 4 °C for 24 h in the dark. Absorption was measured at 664 nm and 647 nm and the levels were calculated after the following formulae (Moran 1982):

$$Chl\ a = 12.64 \cdot A_{664} - 2.99 \cdot A_{647};$$

$$Chl\ b = -5.6 \cdot A_{664} + 23.26 \cdot A_{647};$$

$$Chl\ tot = 7.04 \cdot A_{664} + 20.27 \cdot A_{647}$$

Chl a – chlorophyll a, *Chl b* – chlorophyll b, *Chl tot* – total chlorophyll levels

Results were expressed as mg g⁻¹ FW of the plant material. Measurements were performed three times with two independent repetitions. Photosynthetic pigments were assayed for 3, and 10 days ABA and non-frozen controls, 6 months after thawing, and for 3, 7 and 10 days ABA and controls, 18 months after thawing.

Determination of enzyme activities

Enzyme extraction was performed after Yuan et al. (2002) as follows: 0.2 g FW of the whole shoots were ground with 0.05 g polyvinylpyrrolidone into fine powder with liquid nitrogen in 4 ml of 100 mM potassium phosphate buffer pH 7.2 containing 2 mM EDTA and 8 mM mercaptoethanol. After centrifugation at 15,000 rpm for 25 min at 4 °C aliquots of the supernatant were immediately used for the below described assays. All spectrophotometric measurements were performed using a Boeco S-22 UV/VIS spectrophotometer (Germany). Enzymatic activities were studied for 3, and 10 days ABA, as well as for non-frozen controls, 6 months after thawing, and for 3, 7 and 10 days ABA and controls, 18 months after thawing.

Phenylalanine ammonia-lyase activity (PAL, EC 4.3.1.24)

Estimation of PAL was performed as follows: 0.1 ml enzyme extract was mixed with 0.25 ml 20 mM phenylalanine (dissolved in 100 mM borate buffer, pH 8.8), 2 ml of 100 mM borate buffer and 1 ml of distilled water were added. Control samples contained the supernatant and buffer instead of phenylalanine. After 30, 60 and 90 min of incubation at 30 °C, absorption was measured at 290 nm. Activity unit was calculated as ΔA = 0.01, equivalent to

the production of 3.09 nmol cinnamic acid (Yuan et al. 2002).

Catalase activity (CAT, EC 1.11.1.6)

Estimation of CAT activity was performed at room temperature in 2 ml of reaction mixture, containing 100 mM potassium phosphate buffer pH 7.0, 15 mM H₂O₂ and 0.01 ml supernatant. The reaction was initiated by the addition of H₂O₂ and its decomposition was recorded by the decline of absorbance at 240 nm for 3 min, extinction coefficient 39.4 mM⁻¹ cm⁻¹ (Nelson and Kiesow 1972).

Glutathione reductase activity (GR, EC 1.6.4.2)

Determination of GR was performed at room temperature in 2 ml of reaction mixture, containing 300 mM potassium phosphate buffer pH 7.5, 3 mM MgCl₂, 0.1 mM EDTA, 10 mM GSSG, 0.15 mM NADPH and 0.04 ml supernatant. The reaction was initiated with the addition of NADPH. Extinction change was monitored at 340 nm at a 15 s interval within 3 min, extinction coefficient 6.2 mM⁻¹ cm⁻¹ (Sherwin and Farrant 1998).

Ascorbate peroxidase activity (APX, EC 1.11.1.11)

Determination of APX activity was performed at room temperature in 2 ml reaction mixture consisting of 50 mM potassium phosphate buffer pH 7.0, 0.5 mM AsA (ascorbic acid), 0.1 mM H₂O₂ and 0.04 ml supernatant. Reaction was initiated with the addition of AsA. Change in absorbance was monitored at 290 nm at a 15 s interval within 3 min, extinction coefficient 2.8 mM cm⁻¹ (Nakano and Asada 1981).

All enzymatic activities were expressed per milligram protein. The total protein content was measured by the method of Lowry et al. (1951) using a calibration curve performed with bovine serum albumin.

Non-enzymatic antioxidant assays

For the low molecular antioxidants extraction, 0.2 g of FW of the whole shoots were ground into fine powder with liquid nitrogen, then 5 ml 1 M HClO₄ were added. After 25 min centrifugation at 15 000 rpm at 4° C, the supernatant was placed on ice and pH was adjusted to pH 7 (for glutathione) and pH 6 (for ascorbate) with 5 M K₂CO₃. The potassium perchlorate was removed by further centrifugation and the clear supernatants were used for the assays (Doulis et al. 1997). Non-enzymatic antioxidant enzymes were studied for 3, and 10 d ABA and non-frozen controls, 6 months after thawing, and for 3, 7 and 10 d ABA and controls, 18 months after thawing.

Determination of oxidized (GSSG) and reduced (GSH) glutathione

The concentrations of reduced and oxidized glutathione were determined with an enzyme recycling assay (Griffith 1980). The assay was based on sequential oxidation of glutathione by 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and reduction by NADPH in the presence of GR. For determination of the quantities of GSH plus GSSG and GSSG separately, the extract was processed and subsequently assayed as per the method given earlier (Fadzilla et al. 1997). One sample was used to determine the concentrations of GSH and GSSG and another one—pretreated with 2-vinylpyridin (>99 %, HPLC grade) for masking GSH by derivatization and allow determination of GSSG alone. In each case the assay mixture in 1 ml contained 125 mM potassium phosphate buffer and 6.3 mM EDTA pH 6.5, 0.3 mM NADPH, 3 mM DTNB and 0.01 ml of the supernatant. The reaction was initiated by the addition of 10 µl of GR (5 U/ml) and the change in absorbance at 412 nm was recorded. Standard curves were generated with reduced and oxidized glutathione. The results were expressed per gram FW.

Determination of AsA (ascorbic acid) and DHA (dehydroascorbic acid)

AsA was estimated as the decrease in absorbance for 1 min at 265 nm, in a reaction mixture, consisting of 100 mM potassium phosphate buffer, pH 5.6, 0.2 mM AsA, 5 µl ascorbate oxidase and 0.02 ml supernatant. The reaction was initiated with the addition of the supernatant. DHA was determined in a reaction mixture, consisting of 100 mM potassium phosphate buffer, pH 8.5, 0.2 mM AsA and 10 mM GSH. The reaction mixture was incubated in a water bath for 15 min at 25 °C. The reaction was initiated with the addition of 0.02 ml of the supernatant and the decrease of the absorption of samples was recorded at 265 nm. Standard curves were generated with AsA and DHA (Foyer et al. 1983). The results were expressed per gram FW.

Statistical analyses

All values reported in this work are mean of at least three independent experiments. The mean values ± SE (standard error of the mean) and exact number of experiments are given in the figures and tables. The significance of differences between the control and each treatment was analyzed by Fisher LSD test ($p \leq 0.05$) after performing ANOVA multifactor analysis.

Table 1 Effect of 0.076 μM ABA pre-culture period on *H. rumeliacum* 6 months after cryostorage, thawing and regeneration

Parameter studied 6 months after thawing	Non-frozen control	3 days ABA	10 days ABA
Phenolics [mg g^{-1} DW] (n = 9)	311.1 (± 29.26) ^b	351.6 (± 25.7) ^b	268.2 (± 6.6) ^a
Flavonoids [mg g^{-1} DW] (n = 9)	76.8 (± 5.6) ^a	95.3 (± 3.02) ^c	60.8 (± 3.22) ^a
PAL [nmol mg^{-1} prot min^{-1}] (n = 6)	59.8 (± 4.32) ^c	38.05 (± 0.28) ^b	32.05 (± 0.3) ^a
CAT [nmol mg^{-1} prot min^{-1}] (n = 6)	3.43 (± 0.16) ^a	36.25 (± 8.23) ^c	13.07 (± 0.85) ^b
GR [nmol mg^{-1} prot min^{-1}] (n = 6)	3.95 (± 1.09) ^a	12.52 (± 3.9) ^b	4.79 (± 0.5) ^a
APX [nmol mg^{-1} prot min^{-1}] (n = 6)	33.49 (± 1.2) ^a	37.61 (± 1.12) ^b	33.09 (± 3.91) ^a
MDA [mmol g^{-1} FW] (n = 6)	0.197 (± 0.005) ^b	0.19 (± 0.005) ^b	0.169 (± 0.001) ^a
H ₂ O ₂ [mmol g^{-1} FW] (n = 6)	15.46 (± 0.14) ^a	16.65 (± 0.045) ^a	18.85 (± 0.21) ^b
GSH/GSSG (n = 6)	0.362 (± 0.02) ^b	0.482 (± 0.1) ^c	0.284 (± 0.03) ^a
AsA/DHA (n = 6)	1.6 (± 0.03) ^b	1.9 (± 0.24) ^c	0.78 (± 0.04) ^a
FW/DW (n = 4)	5.16 (± 0.4) ^a	5.55 (± 0.2) ^a	5.19 (± 0.6) ^a
Chl a [mg g^{-1} FW] (n = 6)	0.92 (± 0.08) ^{ab}	0.75 (± 0.06) ^a	1.3 (± 0.09) ^c
Chl b [mg g^{-1} FW] (n = 6)	0.54 (± 0.03) ^b	0.43 (± 0.001) ^a	0.87 (± 0.06) ^c
Chl tot [mg g^{-1} FW] (n = 6)	2.44 (± 0.2) ^b	1.97 (± 0.09) ^a	3.29 (± 0.3) ^c
Chl a/b (n = 6)	1.68 (± 0.04) ^{ab}	1.74 (± 0.1) ^b	1.42 (± 0.2) ^a

Values are mean \pm SE (n is specified for each parameter; $p \leq 0.05$). The same letters denote non-significant differences

Results

Effect of ABA pre-culture duration on the polyphenolics accumulation in *H. rumeliacum* shoots regenerated after cryostorage

Phenolic and flavonoid contents

Six months after thawing phenolic and flavonoid amounts were higher in the 3 days ABA regenerants, in comparison to 10 days ABA ones (Table 1). After 18 months in vitro maintenance, however, total phenolics and flavonoids increased in all three lines of regenerants in comparison with their non-frozen control, with 3 days ABA showing lower levels than 7 and 10 days ABA plants (Fig. 1). It should also be mentioned, that a tendency of decreased phenolic and flavonoid productivity with the prolonging of the subculture period to 18 months was generally observed for both the non-frozen control and 3 days ABA plants (Table 1; Fig. 1).

Hypericins levels

Storage in liquid nitrogen at -196°C did not impair the biosynthetic capacity of in vitro cultured *H. rumeliacum* in terms of condensed anthraquinones biosynthesis 18 months after regeneration (Fig. 2). Moreover, while in 10 days ABA regenerated lines, the levels of hypericin and pseudohypericin did not vary significantly in comparison to the control plants, for 3 and 7 days ABA lines hypericins were significantly higher in comparison to the non-frozen controls. For the 7 days ABA pretreated plants hypericin

amount was increased more than twice in comparison to the controls. The hypericin/pseudohypericin ratio in this *Hypericum* line was also significantly higher in comparison to control, 3 and 10 days ABA pretreated plants (Fig. 2).

Effect of pre-culture duration on the physiological status of plants, regenerated after cryostorage

MDA and H₂O₂ levels

Six months after thawing, MDA and H₂O₂ of 3 and 10 days ABA regenerants did not exceed the ones of the non-frozen control (with only elevated H₂O₂ in 10 days ABA) (Table 1). After 18 months of subculture, however, values of both parameters (Fig. 3) were elevated in the regenerants as compared with non-frozen *H. rumeliacum* (with the exception of the lower MDA in 3 days ABA regenerants). It has to be noted that prolonged tissue culture generally lowered H₂O₂ in the controls and MDA in all groups of plants, in comparison to the 6 months cultivated ones, while H₂O₂ levels remained commensurable for the regenerants during prolonged subculture (Table 1; Fig. 3).

Photosynthetic pigments and dry weight accumulation

Six months after thawing dry weight accumulation was commensurable for both 3 and 10 days ABA regenerants and the non-frozen controls (Table 1). After prolonged subculture, only 3 days ABA regenerants exhibited a slight increase of dry weight accumulation, while the rest of the lines still had commensurable values for this parameter (Table 2). The ABA pre-culture period was also shown to

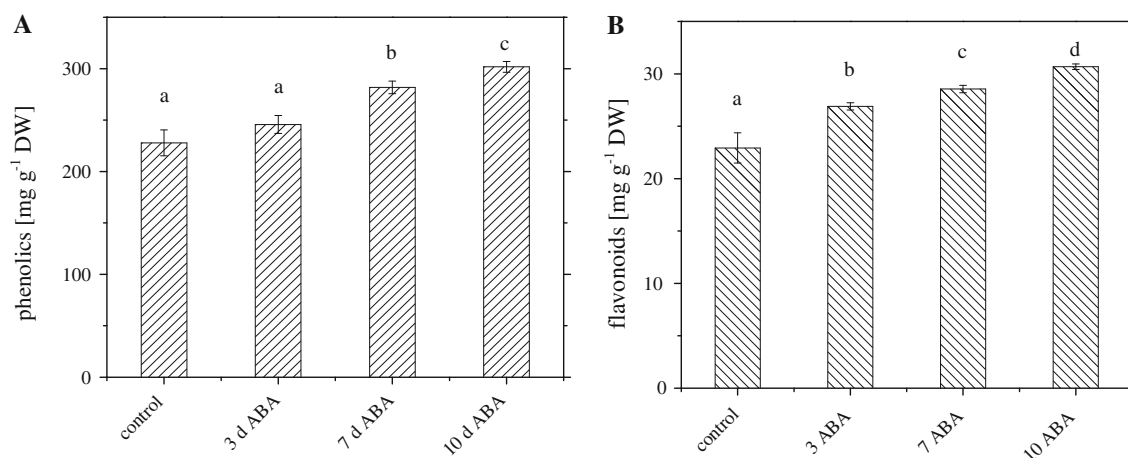


Fig. 1 Impact of ABA pre-culture duration on the phenolic (a) and flavonoid b content of *H. rumeliacum* 18 months after thawing in comparison with the non-frozen control. Values are mean \pm SE ($n = 9$; $p \leq 0.05$). The same letters denote non-significant differences

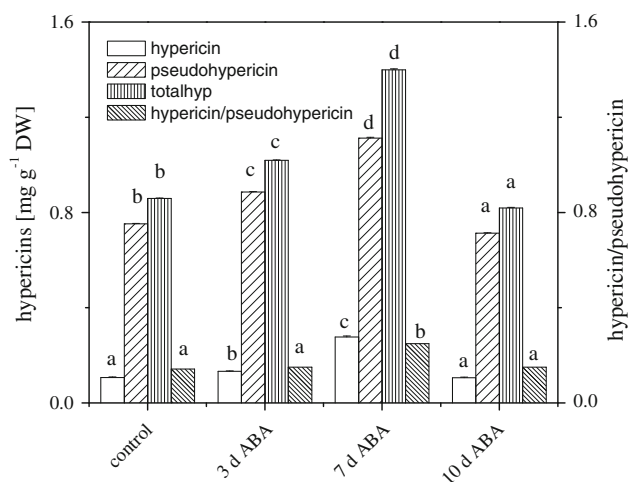


Fig. 2 Impact of ABA pre-culture duration on hypericins levels of *H. rumeliacum* 18 months after thawing in comparison with the non-frozen control. Values are mean \pm SE ($n = 3$; $p \leq 0.05$). The same letters denote non-significant differences

affect the photosynthetic pigments levels in a similar way not only shortly after thawing, but also after prolonged maintenance of the regenerants. Thus, 3 days ABA (characterized with highest oxidative stress prior to dehydration) exhibited lowest levels for chl a, chl b, as well as for total chlorophylls (chl tot), both in short and long-term of maintenance after thawing (Tables 1 and 2). Respectively, 10 days ABA and 7 days ABA exhibited increased or commensurable levels for these parameters with the control. The chl a/b ratio, however was lowest in 10 days ABA regenerants both shortly after thawing and after long-term storage. Noteworthy is the increase of chl a/b in 7 days ABA lines in comparison to 3 and 10 days ABA, as well as control plants 18 months after thawing (Table 2).

Effect of ABA pre-culture duration on the enzymatic activities in *H. rumeliacum* shoots regenerated after cryostorage

PAL activity

Six months after thawing, PAL activity was significantly higher in the 3 days as compared with the 10 days ABA regenerants (Table 1). Both groups displayed lower values than the non-frozen control. After prolonged subculture, no significant difference was found for this parameter between the three groups of regenerants, indicating the restoration of PAL activity (Fig. 4a). Also as compared with the non-frozen control, only 7 days ABA showed slightly lower PAL activity. Noteworthy is also the tendency of a drop of PAL activity in the non-frozen control and as well as in 3 days ABA plants with the prolonging of subculture period to 18 months (Table 1; Fig. 4a).

CAT activity

In regenerants, subcultured for 6 months after cryopreservation, CAT activity was considerably higher in 3 days ABA than in the 10 days ABA lines and the control (Table 1). Further, with prolonging of the cultivation time, the pre-culture period seemed to have an important role on the restoration of CAT activity. Thus, 18 months after thawing, 3 days ABA line displayed the lowest CAT activity in comparison to the other lines, and the highest activity of the enzyme was established for the 10 days ABA line (Fig. 4b). Unlike PAL activity, described above, with prolonged subculture of the non-frozen control, its CAT activity markedly increased with the time (Table 1; Fig. 4b).

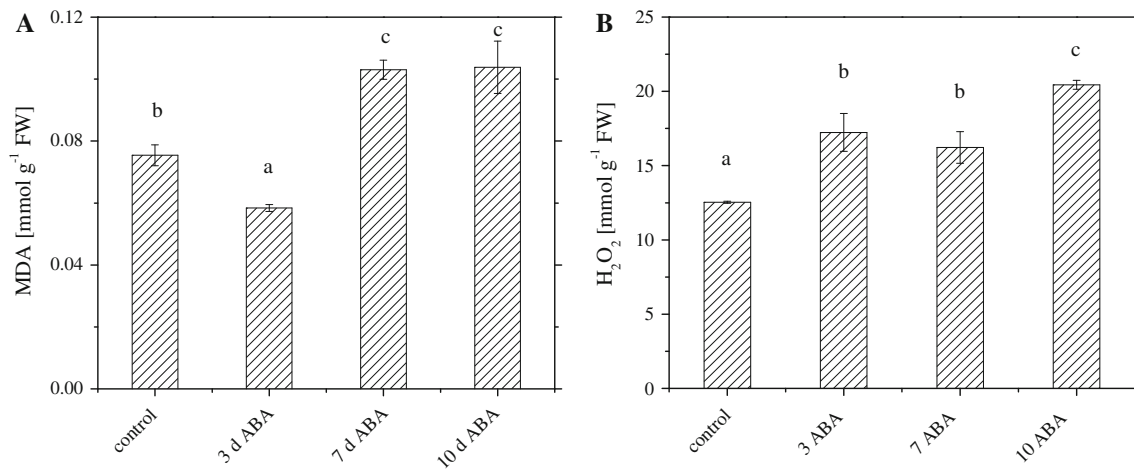


Fig. 3 Impact of ABA pre-culture duration on MDA (a) and H₂O₂ (b) contents of *H. rumeliacum* shoots 18 months after thawing in comparison with the non-frozen control. Values are mean \pm SE (n = 6; $p \leq 0.05$). The same letters denote non-significant differences

Table 2 Effect of ABA pre-culture period on *H. rumeliacum* 18 months after regeneration

Parameter studied 18 months after thawing	Non-frozen control	3 days ABA	7 days ABA	10 days ABA
Chl a [mg g ⁻¹ FW] (n = 6)	1.14 (± 0.1) ^b	0.77 (± 0.09) ^a	1.72 (± 0.2) ^c	1.03 (± 0.05) ^b
Chl b [mg g ⁻¹ FW] (n = 6)	0.36 (± 0.06) ^b	0.29 (± 0.04) ^a	0.32 (± 0.01) ^b	0.58 (± 0.03) ^c
Chl tot [mg g ⁻¹ FW] (n = 6)	1.5 (± 0.07) ^b	1.06 (± 0.1) ^a	2.04 (± 0.2) ^c	1.61 (± 0.09) ^b
Chl a/b (n = 6)	3.45 (± 0.86) ^c	2.71 (± 0.08) ^b	5.26 (± 0.57) ^d	1.79 (± 0.01) ^a
FW/DW (n = 4)	6.04 (± 0.03) ^b	5.32 (± 0.06) ^a	6.63 (± 1.2) ^b	6.44 (± 0.5) ^b
GSH/GSSG (n = 3)	1.75 (± 0.02) ^d	0.560 (± 0.1) ^a	1.402 (± 0.04) ^c	0.875 (± 0.1) ^b
AsA/DHA (n = 6)	2.314 (± 0.6) ^c	1.374 (± 0.4) ^a	4.323 (± 0.32) ^d	1.967 (± 0.06) ^b

Values are mean \pm SE (n specified for each parameter; $p \leq 0.05$). The same letters denote non-significant differences

GR activity

The activity of GR in regenerants 6 months after thawing exhibited behavior similar to the one of CAT. GR activity was elevated in comparison to non-frozen controls, for both 3 and 10 days ABA pre-cultured plants. The values for 3 days ABA significantly exceeded the ones for 10 days ABA (Table 1). However, after prolonged subculture, GR had dropped in cryo retrieved *H. rumeliacum* in comparison to the controls (Fig. 4c). We did not establish correlation between GR activity and pre-culture duration.

APX activity

Correspondingly to CAT and GR, the third antioxidant enzyme, studied in the present work (APX), showed increased activity for 3 days ABA pre-cultured plants 6 months after thawing (Table 1). Then prolonged 18 months of subculture resulted in a drop of its activity in all three lines of regenerated *H. rumeliacum* in comparison to the control (Fig. 4d).

Effect of ABA pre-culture duration on the GSH/GSSG and AsA/DHA levels in *H. rumeliacum* shoots regenerated after cryostorage

While 6 months after thawing, highest GSH/GSSH and AsA/DHA ratios were observed in 3 days ABA pre-cultured lines (Table 1), after prolonged subculture, the same line of regenerants showed the lowest values for both parameters (Table 2). Highest levels of the two parameters were observed for non-frozen controls and 7 days ABA lines.

Discussion

The applicability of cryopreservation protocols to a broad range of genotypes is a key issue for genebanks (Kim et al. 2006). However, as reviewed by Harding et al. (2009), while a number of genotypes held in cryobanks respond satisfactory to cryopreservation, the application of the same successful protocol to others results in low survival. For example, physiological factors have been shown to

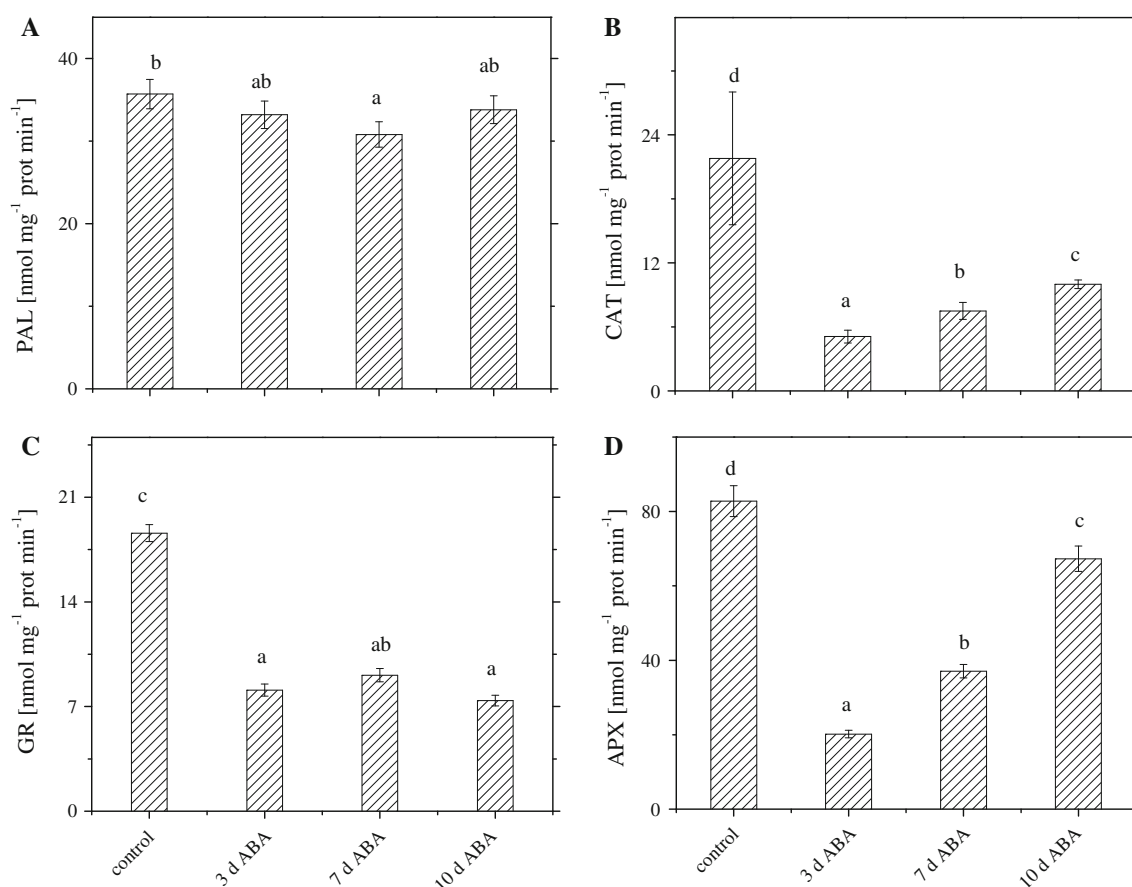


Fig. 4 Impact of ABA pre-culture period on PAL (a), CAT (b), GR (c) and APX (d) activities of *H. rumeliacum* shoots 18 months after thawing in comparison with the non-frozen control. Values are mean \pm SE ($n = 6$; $p \leq 0.05$). The same letters denote non-significant differences

influence survival and development after cryostorage of potato and *Ribes*, even sometimes independently of the protocol and genotype itself. The authors have outlined that a more holistic approach is needed in order to understand the basis of success or failure of cryopreservation of plant germplasm. The comparative study between *Solanum* and *Ribes*, conducted by the authors showed that a wide range of factors determined meristem survival and development following cryopreservation, such as genotype, protocol, culture conditions, medium composition and age of culture. Previous research showed that constitutively high hypericins levels make *H. rumeliacum* a prospective candidate for biotechnological development and long-term preservation of highly productive lines. However, this feature also brings about the problem of coping with the high levels of oxidative stress characteristic for the shoot cultures of the species (Danova et al. 2012). Also, as discussed above, a longer pre-culture period was needed to alleviate injury stress imposed by explant excision preceding pre-culture (Danova 2010). These findings correspond to the report of Roach et al. (2008) of the outburst of reactive oxygen species triggered by the physical injury caused by excision of embryonic axes of *Castanea sativa*. In the present work,

we established that in the shorter term of regeneration (6 months after thawing), 3 days ABA pre-cultured *H. rumeliacum* explants regenerated into plant lines with elevated phenolic and flavonoid levels and correspondingly increased PAL activity as compared with 10 days ABA ones (Table 1). The levels of antioxidant enzymatic activity, as well as the ratios of non-enzymatic antioxidant metabolites GSH/GSSG and AsA/DHA were also raised in 3 days ABA plants, thus contributing to the decrease of MDA and H₂O₂ levels (Table 1). On the contrary, 10 days ABA pre-cultured plants displayed a drop of phenolics and flavonoids and lower PAL activity, as well as lower activity of the three studied antioxidant enzymes and GSH/GSSG and AsA/DHA ratios as compared with 3 days ABA (Table 1). The obtained results corroborate with literature data on the mechanisms of overcoming environmental stress by the plant organism. Stimulated phenolic accumulation together with increased hydroxyl radical activity and enhanced antioxidant status after regeneration, have been observed in the tolerant to cryopreservation *Ribes niger* in comparison to the sensitive *R. ciliatum* (Johnston et al. 2007). In another study on the recovery of *Medicago sativa* after chilling stress, the MDA content decreased due

to the increase of phenolic compounds, which suppressed lipid peroxidation (Bafeel and Ibrahim 2008). During the recovery period, activities of CAT, ascorbate peroxidase (APX) and glutathione reductase (GR) increased significantly, which could possibly restrict the generation of reactive oxygen species associated with chilling stress (Bafeel and Ibrahim 2008). Interestingly, the obtained results in the present work indicate that the physiological state of explants entering into cryopreservation affects the status of regenerants in a time dependent manner. Though in the short-term recovery, regenerants of explants entering cryopreservation with highest oxidative stress displayed an increase of the antioxidant defense, after the prolonged recovery, a drop of these parameters was observed for the same group of plants (Table 2; Fig. 4b–d). Thus, after prolonged culture, unlike in the shorter recovery, the status of the enzymatic antioxidant defense in regenerants was reversely related to the extent of oxidative stress, imposed on *H. rumeliacum* explants during pre-culture. Our results are consistent with the ones of Lynch et al. (2011), who established that optimal pretreatment prior to cryoprotection and controlled cooling could, in part, increase tolerance to cryo-stress by enhancement of endogenous antioxidants (particularly GR), proline and sugars. This observation is of practical importance for the long-term cultivation and potential long-term utilization of specific traits of selected lines, retrieved after cryostorage.

Another important observation is the status of the non-frozen *H. rumeliacum* in the long-term maintenance of the culture. With the time, we observed a marked increase of the antioxidant enzymatic activities, GSH/GSSG and AsA/DHA (Tables 1, 2; Fig. 4b–d). Hence, regardless of the drop of PAL, phenolics and flavonoids, a considerable drop of oxidative stress markers (Fig. 3) and a raise of chl a/b was also observed in 18 months, implying of a stabilization of the control cultured line and its physiological status.

Hypericin levels in *H. rumeliacum* were not impaired by cryostorage. Moreover, the 7 days ABA line displayed a considerable raise of hypericin, pseudohypericin and hypericin/pseudohypericin ratio 18 months after thawing (Fig. 2). In a previous research on *H. perforatum* (Urbanová et al. 2006) it was found that the biosynthetic potential of *H. perforatum*, in terms of total hypericins production (determined spectrophotometrically) in vitro, were preserved in the regenerated plants after cryopreservation by slow cooling. Thus the method of cryopreservation of shoot tip meristems is reliable in terms of preservation of valuable indigenous traits such as the biosynthetic capacity of *Hypericum* species. Further research is needed in order to elucidate whether cryostorage itself, or most probably other steps of the complex cryopreservation protocol could be the critical point, leading to the marked stimulation of hypericin and pseudohypericin

production in the 7 days ABA and 3 days ABA pre-cultured *H. rumeliacum* in the present experiment (Fig. 2). Having in mind the strong pro-oxidant activity of hypericin (Karioti and Bilia 2010), it is a question of further research to optimize the balance between the high hypericins productivity, elevated oxidative stress in this species and the possible difficulties which these factors might be causing in cryopreservation. It is well established that free radical-mediated stress has a role in tissue culture recalcitrance (Benson 2000). Hence, measures for oxidative stress prevention should be experimented in the improvement of *H. rumeliacum* cryopreservation protocol.

Conclusion

In conclusion, liquid nitrogen storage does not impair the biosynthetic capacity of in vitro cultured *H. rumeliacum*. However, special measures are needed for managing the injury stress generated during pre-culture of explants, in order to alleviate its long-lasting harmful effect on the physiological state of regenerants. The elevated oxidative stress in vitro, related to the biosynthetic capacity of the active pro-oxidant hypericins, could possibly also impose problems in its successful cryopreservation. Therefore, oxidative stress prevention should be included in the improvement of the cryopreservation protocol of *H. rumeliacum*.

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