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Production of polyphenolic compounds in shoot cultures of *Hypericum* species characteristic for the Balkan flora

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ABSTRACT: This work compares the polyphenolic compounds content in the *in vitro* shoot cultures of *Hypericum rumeliacum, H. tetrapterum* and in the hypericin non-producing *H. calycinum,* which are characteristic for the Balkan flora. In addition, the impact of different vitamin supplementation to culture medium on secondary metabolites is discussed. Though Gamborg's vitamins supplementation improved growth parameters *in vitro*, it resulted in reduced polyphenolics levels in comparison with Murashige and Skoog's vitamins. Preliminary experiments on cryopreservation of *H. rumeliacum* which were previously performed, gave low rates of plant survival. Therefore, as a part of a broader study for improving the approach for cryopreservation of *H. rumeliacum*, phenolics accumulation, total soluble sugars content as well as malondialdehyde and hydrogen peroxide content were studied during preculture treatment prior to dehydratation and liquid nitrogen immersion and results are discussed in this work.

Key words: *Hypericum rumeliacum, H. tetrapterum, H. calycinum,* shoot cultures, polyphenolics, cryopreservation.

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INTRODUCTION

The interest in mass propagation of medicinal plants has distinctly increased for various reasons. Conventional methods of yield of valuable pharmaceuticals from *in situ* accessions are time consuming; plants have a low rate of fruit set and poor seed germination, and are often under protection or threatened with extinction. Therefore the alternative to this situation is the rapid *in vitro* multiplication of plants and their cultivation under special conditions (Bajaj *et al*. 1988). However, in many cell cultures the amount of the desired product that is formed is too low or there is a decrease in biosynthetic capacity during serial subculture and transfer and thus cells are needed in large quantities. In literature there are data that cultures preserved in liquid nitrogen (-196 °C) retain their biosynthetic potential (Bajaj 1988).

In vitro cultures of *H. perforatum* have been shown to be an easily manipulated system, where physiological, biochemical and genetic parameters can be altered in order to increase secondary metabolites yields (Kirakosyan *et al.* 2004). Recently, the method of cryopreservation is used for storage of selected plant cell and tissue lines (Bajaj 1995; Engelmann 1997). Successful cryopreservation of shoot-tip meristems of *H. perforatum* has been performed showing high recovery rates and preserved biosynthetic capacity of the regenerants (Urbanová *et al.* 2002, 2006).

Shoot cultures of *H. rumeliacum* Boiss. and *H. tetrapterum* Fries were previously induced (Danova *et al.* 2007). High levels of phenolic and flavonoid compounds as well as high regeneration capacity of different explants of *in vitro* cultured *H. rumeliacum* were reported (Danova *et al.* 2008, 2010).

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In a common research work with the working group of Prof Eva Čellárová, at the Department of Experimental Botany and Genetics at the Faculty of Science, P.J. Safarik University Kosice, Slovak Republic, initial experiments on cryopreservation by slow cooling and vitrification of the Balkan endemic *H. rumeliacum* were conducted, showing that though the recovery rates were rather low, the levels of malondialdehyde and hydrogen peroxide of the regenerated plants were in commensurable with the unfrozen controls (Danova *et al.* 2009; Danova & Kapchina-Toteva 2009).

The objective of the present work was to compare the polyphenolic secondary metabolite levels in shoot cultures of *H. rumeliacum, H. tetrapterum* and *H. calycinum* and to study the impact of different vitamin supplementations to the basic Murashige and Skoog medium on growth parameters and biosynthetic capacity of shoot cultures of the three species. In order to optimize the cryopreservation protocol, the total phenolics, total soluble sugars levels and physiological status of *H. rumeliacum* were studied in a model system of the preculture treatment conditions previously applied in cryopreservation experiments of this plant species.

MATERIALS AND METHODS

Plant material and culture conditions. Intact plant material of *H. rumeliacum* was collected in August 2006 in the area of Asenova Krepost, 2 km to the south of Asenovgrad above the bench of Asenica River - SOM 163 524. *H. tetrapterum* (July 2006) was collected in the region of West Stara Planina (West Balkan) Mountains- SOM 165 686. *H. calycinum* was collected at the site of ornamental plants arrangements on the territory of the Seaside Garden – Varna in August 2007 - SOM 165 685.

In vitro shoot cultures of *H. rumeliacum*, *H. tetrapterum* and *H. calycinum* were induced from surface-sterilized mono-nodal stem segments of the *in situ* growing mother plants. Sterilization of explants was performed by means of a 30 seconds rinsing with 70% ethanol, followed by 6 minutes immersion in 0.1% $HgCl_2$, followed by triplicate rinsing in sterile distilled water. For *ex situ* axillary shoot induction, the sterilized stem segments of *H. rumeliacum* and *H. calycinum* were transferred to 0.3mg/l BA supplemented Murashige and Skoog medium, and *H. tetrapterum* – on the basal Murashige and Skoog medium without growth regulators supplementation. Shoots were further maintained on Murashige & Skoog (1962) culture medium at temperature 25°C, 16/8 h photoperiod and irradiation intensity of 60 $\mu mol.m^{-2}.s^{-1}$, with a 45-day period of regular subculture.

For the study of impact of vitamin supplementation two media variants were chosen: I – Gamborg's vitamins (Gamborg *et al*. 1968) supplementation to the Murashige and Skoog's medium and II – the basic Murashige and Skoog's culture medium formula. Both media were supplemented with 100mg/l myo-inositol and 3% sucrose, solidified with 0.7% agar and autoclaved at 121°C for 25min. Three-node shoot segments of the *in vitro* growing *Hypericum* species were placed on the two media and cultured for 45 days as described above.

Total phenoics and flavonoids determination. 100mg DW (air dry weight) of the samples of shoot cultures of the three studied *Hypericum* species *in vitro* were extracted with hot ethanol. Total phenolics were determined by the Folin & Ciocalteu's colorimetric method of Singleton *et al.* (1999) with modifications. A 0.050 ml aliquot of the extract was placed in test-tube and 1,700 ml distilled water, 0,250 ml of 1:1 Folin & Ciocalteu's reagent (Sigma-Aldrich) and 0,500 ml 20% aqueous Na_2CO_3 were added. The absorbtion was measured at 730nm and the total phenolics were calculated by means of a calibration curve of chlorogenic acid (in the range of 10µg/ml to 100 µg/ml). The result was expressed as milligrams of chlorogenic acid equivalent per gram of DW of the sample.

Total flavonoids content of leaf samples of the plant was measured using a colorimetric assay in accordance to a modification of the method of Zhishen (1999). In brief, 100 mg DW of the samples were extracted with hot ethanol. A 0,100 ml aliquot of the extract was placed in test-tube and 0,900 ml distilled water, 0,060 ml 5% aqueous $NaNO_2$ and 0,060 ml 10% aqueous $AlCl_3$ were added. After the final addition of 0,400 ml 1n NaOH and 0,450 ml distilled water, the absorption at 510 nm was measured and the concentration was calculated using a calibration curve of (+)catechin (in the range of 2 µg/ml to 80 µg/ml). The result was expressed as milligrams of (+)catechin equivalent per gram of DW of the sample.

Total hypericins determination. Total hypericins were extracted and assayed spectrophotometrically as previously described by Urbanová *et al.* (2006) with modifications. 100mg DW of shoot culture material of *H. rumeliacum* and *H. tetrapterum* were homogenized and macerated for 30 min at room temperature with chloroform, followed by 20 minutes of ultrasonic extraction. After discarding of chloroform phase, addition of fresh chloroform and new 48 hours of maceration, repetition of the ultrasonic extraction was performed, chloroform extract discarded and the defatted plant material was repeatedly extracted with fresh portions of methanol in an ultrasonic bath until discoloration of the solvent. The combined methanolic extracts were evaporated *in vacuo* at 40°C and after filtration adjusted to a set volume. The absorbance of samples was measured at 590nm. Total hypericins content was calculated as relative absorption units per gram DW.

Table 1. Some morphometric characteristics of the three *Hypericum* species in the two media variants (I – Gamborg's vitamins and II – MS vitamins). **For H. tetrapterum and H. calycinum characteristics are taken only for axillary shoots longer than 0.7cm.* Standard deviations of three separate measurements are marked in brackets.

Plant species/medium variant	FW/DW ratio	Shoot length* [cm]	Number axillary shoots per explant	Number of leaf couples per shoot*	Number of leaf couples per 1 cm
H. rumeliacum I	4.81 (± 0.33)	2.03	3.3	3.75	1.84
H. rumeliacum II	5.02 (± 0.29)	1.69	2.35	3.79	2.24
H. tetrapterum I	4.71 (± 0.27)	5.4	2.56	5.83	1.07
H. tetrapterum II	4.97 (± 0.22)	4.38	2.53	5.16	1.17
H. calycinum I	4.75 (± 0.31)	2.1	2	4	1.9
H. calycinum II	4.7 (± 0.23)	2.23	1.94	4.33	1.94

Preculture model system experiment with (i) malondialdehyde and hydrogen peroxide, (ii) total sugars, (iii) phenolics and (iv) water content/dry mass accumulation determination. Preculture treatment in the cryopreservation protocol is aimed at adapting the metabolism of plant tissues to the stress of freezing. However, before being immersed into liquid nitrogen, plant tissues have to be dehydrated to 20-30% in order to avoid the formation of intracellular ice crystals which are deteriorating to the living cells. Therefore preculture is also aiming to prepare the plant tissues to the stress of dehydration prior to the cooling at ultra-low temperatures of liquid nitrogen (-196°C) (Panis & Lambardi 2005; http://www.fao.org/biotech/C13doc.htm). Different approaches of preculture treatment are applied in different cryopreservation protocols, reported in literature (Urbanová *et al.* 2002; Panis & Lambardi 2005). The preculture treatment, previously performed for *H. rumeliacum* prior to dehydration and freezing was based on 0.076μM ABA (abscisic acid) treatment of shoot tips in a liquid culture medium, supplemented with 0.5 mg/l BA (6-benzyladenine) for 3, 7 and 10 days periods (Danova *et al.* 2009; Danova & Kapchina-Toteva 2009). Therefore for the preculture model experiment, performed in the present work, shoot tips, isolated from *in vitro* shoots of *H. rumeliacum,* were grown in liquid MS culture medium with Gamborg's vitamins (1968) and 100mg/l myo-inositol, containing 0.5mg/l BA ($RMB_{0.5}$ medium) and supplemented with 0.076μM ABA for 3, 7 and 10 days. Controls were grown on the same liquid $RMB_{0.5}$ medium lacking ABA supplementation. 50 ± 5 explants were used for each group of treatments.

(i) MDA and H_2O_2 determination - 120 mg FW (fresh weight) of plant material was homogenized at 4°C with 0.1 % trichloroacetic acid and centrifuged for 20 min at 15 000 rotations per minute. The level of lipid peroxidation was measured in terms of content of the product of reaction of malondialdehyde (MDA) with thiobarbituric acid, as described by Dhindsa *et al.* (1981) – including thiobarbituric acid addition and a heat/cool cycle, determined spectrophotometrically (λ= 532 and 600nm) and calculated using its extinction coeficient $155mM^{-1}cm^{-1}$. The endogenous level of H_2O_2 (hydrogen peroxide) was determined spectrophotometrically (λ=390nm) after incubation of explant extracts with 1mol/l KI. The content was calculated using a standard curve (Jessup *et al.* 1994).

(ii) Total soluble sugars and (iii) phenolics determination - For the total soluble sugars assay, 100 mg DW of the plant material was homogenized with hot ethanol (60°C) and filtered through glass filter. After addition of distilled water, 5% phenol and concentrated sulphuric acid to an aliquot of the filtrate, absorption was measured at 485nm (Dubois *et al.* 1956). Values were calculated using standard curve of sucrose. The assay for total phenolics was performed as described above.

(iv) Water content/dry mass accumulation - For determination of water content, plant material of up to 250 mg FW per sample were dried at first at air temperature for 5 days and then at 40°C for 48 hours to obtain the air DW and also asses the dry mass accumulation of samples.

RESULTS AND DISCUSSION

Some morphometric characteristics of the three *Hypericum* species *in vitro* are presented in Table 1. As it is seen in the Table, in medium variant I (Gamborg's vitamins) plants are characterized by a higher shoot length and number of axillary shoots formed per explant. The difference of the axillary shoots number formed in the two media variants was most distinct in *H. rumeliacum* in comparison with the two other species.

Within the deviation of the measurements, there is no significant difference in dry weight accumulation in MS medium variant (II) in comparison to Gamborg's vitamin

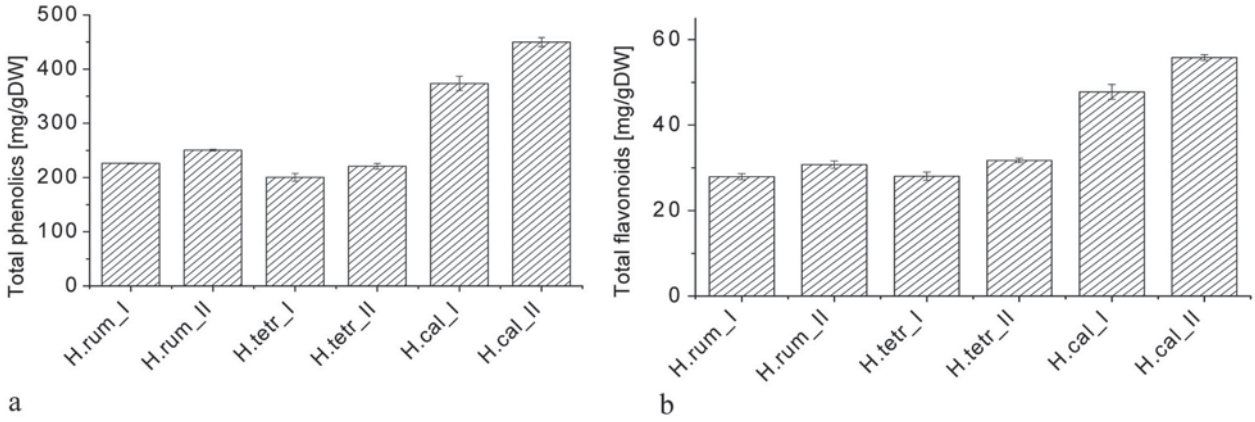

Fig. 1. Total phenolics (a) and flavonoids (b) levels in the three studied *Hypericum* species *in vitro*, in the two media variants(*H. rum – H. rumeliacum, H. tetr – H. tetrapterum and H. cal – H. calycinum; I – Gamborg's vitamin supplementation and II - MS vitamins supplementation*). Vertical bars denote standard deviation of three repetitions of two independent measurements.

supplemented medium (variant I). Interestingly, the number of leaf couples per cm of shoot length in media variants I is lower than the second variant implying of a tendency of stem tissue predominance in Gamborg's vitamin supplemented culture media.

Secondary metabolites levels in shoot cultures of the three studied species. Total polyphenolic compounds were assayed in the shoot cultures of the three studied *Hypericum* species in the two media variants, in order to compare their potential as a source for laboratory yield of valuable compounds with antioxidant properties. The results are presented in Figure 1. While the levels of these metabolites are commensurable for *H. rumeliacum* and *H. tetrapterum*, their content in the shoot cultures of *H. calycinum* is significantly higher compared to the other two species *in vitro.* Therefore *H. calycinum* is a favorable candidate for *in vitro* producer of secondary metabolites with phenolic and flavonoid nature. Comparison of the total phenolics and flavonoids levels between the three species *in vitro* for the two media variants show that though variant I (Gamborg's vitamins) improved growth parameters *in vitro* it has the inverse influence as far as secondary metabolites with phenolic structure are concerned. In Figure 2, it is seen that the levels of total hypericins, expressed as relative units of absorbance at 590nm per gram DW are dramatically higher in *H. rumeliacum* in comparison to *H. tetrapterum.* Though phenolic and flavonoid compounds were in commenrurable levels for these two species, the high levels of total hypericins in *H. rumeliacum in vitro,* make this species an interesting object as far as hypericin production *in vitro* is concerned. The plants cultured in medium variant II display slightly higher levels of total hypericins. However, it is important to consider the overall low levels of this metabolite and keep in mind that the method for

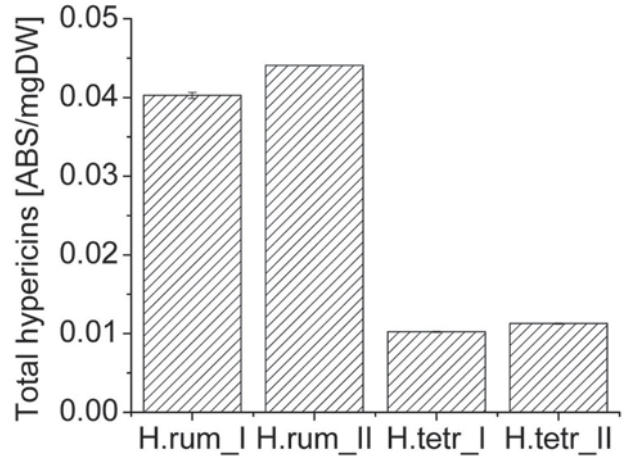

Fig. 2. Total hypericins content in *H. rumeliacum (H. rum)* and *H. tetrapterum (H. tetr)* grown in the two media variants *in vitro (I – Gamborg's vitamins, II - MS vitamins).*

spectrophotometric determination at this wavelength is also sensitive to other compounds as anthocyanidins and photosynthetic pigments, which could also be present in the defatted methanolic extract. Therefore a further detailed phytochemical research is planned in order to elucidate the hypericin and pseudohypericin levels and ratio of the studied species in the two media variants *in vitro.*

Preculture experiments. The survival rates for cryopreservation of *H. rumeliacum* by vitrification as previously reported (Danova *et al.* 2009; Danova & Kapchina-Toteva 2009) were rather low (up to 2.2%) when compared to the successful protocol reported

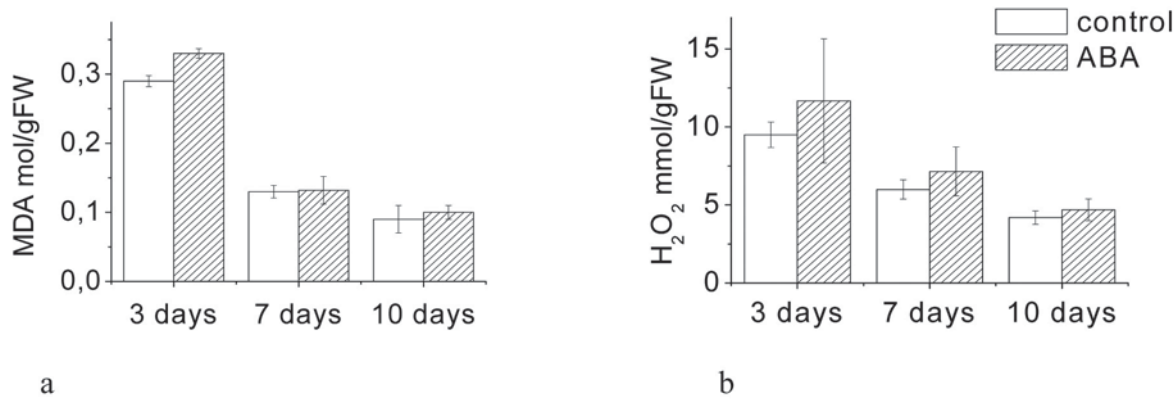

Fig. 3. Lipid peroxidation (a) and oxidative stress (b) of *H. rumeliacum* shoot tips placed in $RMB_{0.5}$ liquid medium lacking ABA ("control") and 0.076μM ABA supplemented ("ABA") maintained for 3, 7 and 10 days at the experimental preculture conditions of the previously performed cryopreservation protocol of the species. The vertical bars denoting standard deviation of three measurements.

for *H. perforatum*, performed by similar procedures by Urbanová *et al*. (2002). The low survival rates of *H. rumeliacum* when subjected to the similar preculture and freezing conditions as *H. perforatum*, implies that there are genotype-specific peculiarities leading to the poorer response and there is need to adjust the existing protocol to the specific peculiarities of *H. rumeliacum*. Therefore it is important to study the specific physiological and biochemical behavior of the species during the consecutive steps of the cryopreservation protocol in order to better understand its specific response and adjust the procedure in order to obtain higher survival rates.

(i) Lipid peroxidation (MDA) and oxidative stress (H_2O_2) were monitored during the preculture conditions of the above described experimental model, designed to repeat the conditions of ABA preculture, previously applied for *H. rumeliacum* and compare the status of "ABA – treated" with control "ABA-non treated" shoot tips. The purpose of this experiment was to assess the effectiveness of ABA supplementation to the liquid medium, for improvement of the physiological status of treated plant tissues. Therefore comparison was made with untreated controls (lacking ABA in the $RMB_{0.5}$) in order to better understand the difference in the processes, occurring in *H. rumeliacum* shoot tips in a liquid $RMB_{0.5}$ culture media supplemented with 0.076 μM ABA (as in the cryopreservation protocol) compared to a control lacking ABA. The experimental results indicate that the overall MDA (Figure 3a) and H_2O_2 (Figure 3b) levels decrease during prolonged preculture in the liquid medium for both ABA treated and control variants. This behavior is logical as during the longer preculture period shoot-tip explants recover from the stress of mechanical injury imposed on them during cutting and transfer to the liquid media for preculture. This is in agreement with the dramatic increase in the survival rate with increase of preculture period in cryopreservation by slow cooling that is previously reported for *H. perforatum* by Urbanová *et al.* (2002). The authors report therein on cryopreservation of 5 different genotypes of *H. perforatum* by slow cooling. Pregrowth was performed in $RMB_{0.5}$ liqid medium with 0.5M manitol, 0.76 μM and 0.076 μM ABA. The 3 days 0.076 μM ABA preculture resulted in 3% survival rate in only one of the genotypes and was not successful for the others. Prolonging the preculture period to 10 and 14 days however significantly increased the survival rate up to 48 and 45 % respectively and was not successful for only one of the genotypes in each case (Urbanová *et al.* 2002).

(ii) Total soluble sugars levels (Figure 4a) in the preculture model system for *H. rumeliacum,* performed in this work, were monitored as a marker for the capacity of ABA in the above described preculture conditions to contribute to reserve substances accumulation during such treatment, as well as to better understand the physiological status of the plant tissues during preculture in the liquid medium system of the previously experimented cryopreservation protocol. Photosynthesis provides plants with sugars that play a central role in the plant life cycle as energy sources, storage molecules, structural components or intermediates for the synthesis of other organic molecules. Moreover significant overlapping between ABA and glucose signaling has been recently reported (Dekkers *et al*. 2008). The results of the present study show that overall total soluble sugars accumulation has a lag phase before their accumulation begins in the tissues in both ABA treated and control samples. Unexpectedly, sugar levels in ABA treated samples are even lower than in the untreated controls. This observation might be explained with an inhibitory effect of ABA on the photosynthetic capacity and carbon assimilation (Seeman & Sharkey 1987). With the prolonging of preculture period this ratio is being

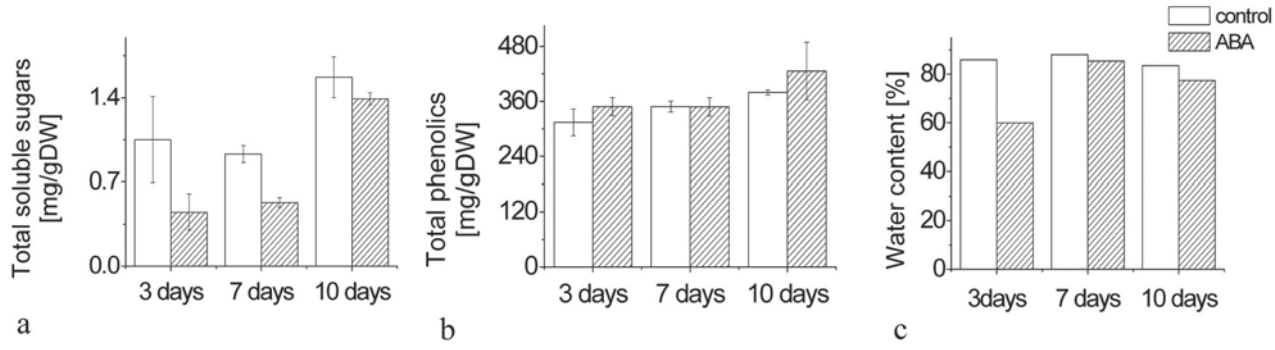

Fig. 4. Total soluble sugars (a), phenolics (b) and water content (c) in ABA treated and control shoot tips of *H. rumeliacum* in liquid $RMB_{0.5}$ medium. The vertical bars denoting standard deviation of three measurements.

"compensated" for ABA treated plants compared to their controls, which might be due to depletion of exogenous ABA in the liquid medium.

(iii) Total phenolics levels - While for the purposes of utilization of plant cell and tissue culture for the yield of diverse compounds with phenolic structure is beneficial (their high levels are a desired feature), the excessive phenolic accumulation adversely affects the successive outcome of cryopreservation. Therefore the levels of phenolics during the stage of preculture prior to cryopreservation are also studied in this work. There is slight general increase of total phenolics levels during preculture for ABA treated and control explants, and the comparison between both groups does not seem to be sizable (Figure 4b). However, having in mind the high biosynthetic capacity of *H. rumeliacum* as a producer of compounds with phenolic structure *in vitro*, demonstrated above, this factor has also to be taken into consideration for further optimization of the protocol for cryopreservation.

(iv)The water status of explants entering the freezing step is a crucial point in the cryopreservation protocol. Prior to the step of liquid nitrogen immersion, plant tissues must be dehydrated. The physiological role of dehydration is to reduce intracellular water content and minimize intracellular ice crystal formation and tissue damage during freezing/thawing stages. Dehydration needed for cryopreservation is water content reduction to at least 20-30% which most hydrated tissues can not withstand. The key for successful cryopreservation is thus shifted from freezing tolerance to dehydration tolerance (Panis & Lambardi 2005). The preculture step in a protocol is applied to ensure the survival of explants during the dehydration imposed by cryoprotection prior to freezing.

In controlled slow cooling protocols, the dehydration is accomplished due to the extra-cellular ice formation and water osmotically "leaving" the cell towards the increasingly concentrating extra cellular solution. In vitrification protocols where tissues are rapidly immersed into liquid nitrogen, the dehydration is imposed osmotically through diverse chemical cryoprotectant solutions. In shot, in the previously described vitrification protocol applied to *H. rumeliacum,* shoot tips were dehydrated by means of placing into a loading solution (LS) containing 2 M glycerol and 0.4 M sucrose at room temperature and after 20 minutes the shoot tips were transferred to sterile cryotubes containing plant vitrifying solution (PVS3) (50% w/v sucrose, 50% w/v glycerol) and equilibrated on ice for 90 minutes. After the equilibration step, the samples were immersed directly in liquid nitrogen (Danova *et al.* 2009; Danova & Kapchina-Toteva 2009).

The results of water content measurement during preculture (Figure 4c) indicate inhibition of water accumulation in ABA treated plants vs. their control only at the beginning of the preculture period. Hydration of ABA treated explants is restored with the time of culture in the liquid medium. The last steps before the direct immersion into liquid nitrogen – LS and PVS3 treatments (Danova *et al.* 2009; Danova & Kapchina-Toteva 2009) which provide the dehydration of the tissues prior to freezing are not concerned in this work. Further research is planned to elucidate the actual percentage of dehydration caused by the LS and PVS treatment in order to asses the actual physiological role of the preculture in this protocol to prepare the explants for dehydration. Comparison of survival rates and physiological behavior of different species of the *Hypericum* genus when undergoing the same treatments would give valuable information on the genotype-specific properties leading to differences in effectiveness of the same protocol and would make the procedure less empirical. The in-depth understanding and relevant interpretation of the physiological processes taking place during the different steps of cryopreservation protocol will help to successfully apply this technique also to other species.

CONCLUSIONS

This work compares the biosynthetic capacity of three *Hypericum* species characteristic for the Balkan region to produce antioxidant secondary metabolites with phenolic structure in laboratory conditions. While commensurable levels of total phenolics and flavonoids were found for *H. rumeliacum* and *H. tetrapterum,* the first species was shown to produce dramatically higher total hypericins than the second one. The non-hypericin producing *H. calycinum* is a very favourable producer for total phenolic and flavonoid compounds in comparison with the other two species *in vitro.*

The results, obtained by the study on the impact of vitamin supplementation in the media to the polyphenolic compounds levels *in vitro*, are in agreement with the concept of the needed balance between the biomass production and secondary metabolites content for the achievement of an effective yield of secondary metabolites by the methods of plant biotechnology.

As a part of a planned more detailed research, preliminary results on the physiological behavior and total phenolics and sugars content of *H. rumeliacum* shoot tips during preculture treatment, prior to dehydratation and liquid nitrogen immersion, are reported here. Further research is needed in order to elucidate the dehydration occurring at each step prior to freezing, as well as a more detailed study of the biochemical and physiological processes occurring at the different steps prior to freezing to be able to understand their effectiveness in the preparation of the plant tissues for the dehydration during cryoprotection.

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REZIME

Produkcija polifenolnih jedinjenja u kulturama izdanaka odabranih vrsta roda *Hypericum* sa Balkana

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Ovaj rad daje poredjenje sadržaja polifenolnih jedinjenja u *in vitro* kulturama izdanaka vrsta *Hypericum rumeliacum, H. tetrapterum* i *H. calycinum* (ne produkuje hipericin). Sve navedene vrste javljaju se u flori Balkana. Osim toga, uticaj dodatih vitamina medijumu za gajenje na sastav I sekundarnih metabolite je diskutovan. Dodatak u vidu Gamborgovih vitamina, podsticao je rast dva parametra praćena u *in vitro* uslovima, ali I redukovao nivo polifenola u poredjenju sa normalnim vitaminskim sastavom MS podloge. Preliminarni rezultati u krioprezervaciji *H. rumeliacum* davali su nizak nivo preživljavanja. Stoga je vodjen širi pristup krioprezervaciji *H. rumeliacum*–a, u vidu izučavanja akumulacije polifenola, ukupnih solubilnih šećera, peroksidacije lipida I oksidativnog stresa za vreme pretretmana pre dehidratacije i imerzije u tečni azot. Dobijeni rezultati su diskutovani u ovom radu.

Ključne reči: *Hypericum rumeliacum, H. tetrapterum, H. calycinum,* kultura izdanaka, polifenoli, krioprezervacija