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Influence of vitamins on polyphenolic content, morphological development, and stress response in shoot cultures of *Hypericum* spp

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Abstract Hypericin is a widely studied phytopharmaceutical produced by the more developed representatives of the *Hypericum* genus. We established that Gamborg's (G5) vitamins significantly raised hypericins levels in *H. rumeliacum* and *H. tetrapterum* shoot cultures in comparison with the Murashige and Skoog's (MS) vitamin supplementation. To elucidate this effect, we further examined the impact of the two vitamin formulations on the polyphenolic compounds, morphological development, hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) levels in the two species, as well as in hypericin non-producing *H. calycinum*. It was found that in all three species Gamborg vitamins decreased the polyphenolic compounds content, stimulated multiplication, decreased leaf compactness and increased dark glands density per square millimeter (for the two hypericin producing species) as compared to MS vitamins. In addition, the raised levels of hypericins in *H. rumeliacum* and *H. tetrapterum*, were also connected to increased content of H_2O_2 and MDA in vitro. Moreover, the values of these two parameters were considerably lower in hypericin non-producing *H. calycinum*, implying that oxidative stress either by increased metabolic activity, connected with higher hypericin production, or by a possible auto-toxic effect of the high amounts of pro-oxidant hypericin itself could be induced in *Hypericum* in vitro.

Keywords *Hypericum* shoot cultures · Vitamin supplementation · Morphological development · Hypericin productivity in vitro · Hypericin production and reactive oxygen species in vitro

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Introduction

Hypericin has been widely studied in recent years due to its inherent antidepressant, antiviral and antimicrobial properties, as well as its appreciable pro-oxidant activities and potential as an active photosensitizer in photodynamic therapy of cancer (Karioti and Bilia 2010). Literature data have shown that the evolutionary more developed *Hypericum* species possess an increased capacity of hypericin production as compared to the more primitive sections of the genus. Chemotaxonomic surveys have revealed that some *Hypericum* species, such as *H. boissieri*, *H. barbatum* and *H. rumeliacum* (representatives of *Drosocarpium* section) may contain twofold to fourfold higher amounts of hypericins than *H. perforatum* Karioti and Bilia (2010).

As far as the authors are aware, hypericin production has been intensively studied mainly in cell, tissue and organ cultures of *H. perforatum* and rarely in other representatives of the genus, belonging also to the *Hypericum* section (Kartning et al. 1996; Kirakosyan et al. 2004; Cui et al. 2010; Savio et al. 2012). Surprisingly, except in a recent work comparing the secondary metabolites in *H. hirsutum* (*Taeniocarpium* Jaub. et Spach section) and *H. maculatum* (*Hypericum* section) (Coste et al. 2011), hypericin productivity in vitro has not yet been explored for representatives of other evolutionarily developed sections of the genus.

Along with biotic and abiotic elicitation models (Kirakosyan et al. 2000; Sirvent and Gibson 2002), also media formulations have been broadly investigated regarding their effect on *Hypericum* growth and secondary metabolites productivity in vitro. Modifications comprise growth regulators, inorganic salts variations, based on the individual empirical experience of the tissue culture laboratory (Goel et al. 2009; Palmer and Keller 2011).

In previous work, we established a system for the effective in vitro multiplication of *H. rumeliacum*, allowing for rapid in vitro biomass formation, together with retention of the capacity of polyphenolic compounds production (Danova et al. 2010). Further, polyphenolic compounds were compared spectrophotometrically in *H. rumeliacum* (*Drosocarpium* section), *H. tetrapterum* (*Hypericum* section) and hypericin non-producing *H. calycinum* (*Ascyrea* section) shoot cultures. Hypericins were significanty higher in *H. rumeliacum*, compared to *H. tetrapterum* in vitro. On the other hand, hypericin non-producing *H. calycinum* displayed elevated phenolic and flavonoid compound levels, as compared to the two hypericin producing species (Danova 2010).

It is well known that the secondary metabolite profile is a result of the complex relations of the plant organism with its environment. Understanding the key features connected with the biosynthesis of desired secondary metabolites would provide useful tools for their targeted production in an evidence-based rather than empirical manner. Thus the aims of the present work were: (1) to study whether in vitro culture development alters the polyphenolic profiles by comparison of the wild growing accessions of *H. rumeliacum*, *H. tetrapterum* and *H. calycinum* and their shoot cultures; (2) to search for possible relations between the morphological development and the polyphenolic compounds accumulation in *H. rumeliacum*, *H. tetrapterum* and *H. calycinum* shoot cultures; (3) to evaluate the impact of hypericin levels on the stress markers (H_2O_2 and MDA) in vitro by comparing hypericin high producing *H. rumeliacum*, hypericin low producing *H. tetrapterum* and hypericin non-producing *H. calycinum*.

Materials and methods

Plant material and culture conditions

Wild growing *Hypericum rumeliacum* Boiss., *Hypericum tetrapterum* Fries. and *Hypericum calycinum* L. were collected in Bulgaria and shoot cultures were initiated from surface sterilized stem segments of the aerial parts as previously described (Danova 2010; Danova et al. 2010).

In order to study the effect of modified vitamin supplementation, two media were tested, as both were supplemented with the basic Murashige and Skoog's (1962) macro- and microsalts, 100 mg l^{-1} myo-inositol, 2 mg l^{-1} glycine and 3 % sucrose. The two media differed in vitamin supplementations as follows: I—Gamborg et al.'s (1968) vitamins and II—MS vitamins formulation. Media were solidified with 0.7 % agar and autoclaved at 121 °C for 25 min. Three-node shoot segments of the three *Hypericum* species were placed in the two media and subcultured in every 45 days for three consecutive periods at 25 °C, 16/8 h photoperiod at cool white light.

Morphometric characterization

Fresh/Dry weight ratio (FW/DW)—fresh plant material was divided into four portions of 0.3 g each and dried at 40 °C until constant weight. The FW/DW was calculated for each individual portion and the average value is presented.

Index of compactness (IC)—the parameter was calculated using the formula IC (leaf couples per cm) = average number of leaf couples per shoot / average shoot length (cm) (Bertoli et al. 2008).

Hypericin glands number (HGN)—both leaves per couple were collected from 30 separate shoots of each *Hypericum* species. Leaves were collected from three stem zones—basal, medium and apical. The number of hypericin glands was counted for each examined leaf and the average value is presented for the respective segment.

Leaf area (LA [mm^2]) was measured on a pre-calibrated digital image of each leaf, and the average value is presented in square millimeters for each of the respective stem segments.

Hypericin glands density (HGD = HGN mm^{-2}) was calculated individually for each leaf sample as the number of hypericin glands per square millimeter and the average value of all measurements is presented.

For *H. rumeliacum* and *H. tetrapterum*, morphometric parameters were assessed for plant material of 50, and for *H. calycinum*—of 20 separate growing vessels.

Total phenoics and flavonoids assays

Sample preparation—100 mg DW were extracted with hot ethanol (70 °C) and centrifuged at 15,000 rpm. Aliquots of the supernatant were further used for the polyphenolic contents assays.

Total phenolic compounds were determined by the Folin and Ciocalteu's colorimetric method of Singleton et al. (1999) as follows: a 50 μl aliquot of the extract was placed in a test-tube and 1.700 ml distilled water, 250 μl of 1:1 Folin and Ciocalteu's reagent and 500 μl 20 % aqueous Na_2CO_3 were added. The absorbance was detected at 730 nm and the total phenolics were calculated by means

of a calibration curve of chlorogenic acid (in the range of 30–100 μg ml^{-1}). The result was expressed as μg of chlorogenic acid equivalent per 1 g of DW of the sample.

Total flavonoids were determined using a colorimetric assay by a modification of the method of Zhishen et al. (1999): a 100 μl aliquot of the extract was placed in test-tube and 900 μl distilled water, 60 μl 5 % aqueous NaNO$_2$ and 60 μl 10 % aqueous AlCl$_3$ were added. After the final addition of 400 μl 1n NaOH and 450 μl distilled water, the absorbance at 510 nm was detected and the concentration was calculated using a calibration curve of (+)catechin (in the range of 2–80 μg ml^{-1}).

Plant material of at least 15 separately growing individual plants in situ and from at least 15 separate culture vessels in vitro was used for these assays. Extractions were performed two times independently and measurements performed in three repetitions of the colorimetric reaction per each extraction.

Quantification of hypericin and pseudohypericin

Sample preparation—100 mg DW of plant material were macerated for 30 min at room temperature with chloroform and extracted in an ultrasonic bath for 20 min. After discarding the chloroform phase, the material was covered with fresh chloroform and left for 48 h maceration at room temperature. Then the ultrasonic extraction was repeated, the chloroform extract was removed and the defatted plant material—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 vacuo at 40 °C, filtered and adjusted to 10 ml.

Analytical procedure—hypericin (Hyp) and pseudohypericin (psHyp) were quantified by a modified method for the RP HPLC simultaneous determination of hypericin and pseudohypericin (Häberlein et al. 1992; Krämer and Wiartall 1992; Balogh and Li 1999) by means of 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. A stepwise ternary gradient was employed using ethyl acetate (solvent A), methanol (solvent B) and 0.05 mol/l aqueous K$_2$CO$_3$ (pH 4.0) (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^{-1} 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 6 to 95 μg ml^{-1} hypericin under exactly the same chromatographic conditions. The absorbance was detected at 590 nm.

All measurements were performed in triplicate. The detection limit of hypericin was 1 ng.

MDA and H$_2$O$_2$ determination

120 mg FW were homogenized at 4 °C with 0.1 % trichloroacetic acid and centrifuged for 20 min at 15,000 rpm. MDA content was measured spectrophotometrically at $\lambda = 532$ and 600 nm after addition of thiobarbituric acid and performing a heat/cool cycle (Dhindsa et al. 1981). Extinction coefficient 155 mM^{-1} cm^{-1} was used according to Heath and Packer (1968).

The endogenous level of hydrogen peroxide (H$_2$O$_2$) was determined spectrophotometrically ($\lambda = 390$ nm) after incubation of the plant extracts with 1 mol l^{-1} KI. The content was calculated using a standard curve (Jessup et al. 1994). Two separate assays with three repetitions were performed.

Statistical analyses

The average sample sizes and measurement repetitions are specified in each paragraph of Materials and Methods above. The experimental data for growth parameters: shoot length, axillary shoots formed per explant, and leaf couples per shoot were subjected to frequency analysis. After transformation of values to meet normal assumptions, the means were compared by t test of unequal variances at $P \leq 0.05$. Unless otherwise stated, differences were considered statistically significant at $P \leq 0.05$. Correlation analysis was carried out and relations are presented by the correlation coefficient (r) throughout the text.

Results and discussion

Impact of vitamin supplementation on the growth parameters

Gamborg's vitamins (medium I) increased the shoot length as compared to MS vitamins (medium II). The effect was most expressed in *H. rumeliacum* and insignificant in *H. calycinum* (Figs. 1a–c, 2). Likewise, axillary shoots formation was stimulated by Gamborg's vitamins for hypericin producing *H. rumeliacum* and *H. tetrapterum*, while this effect was insignificant for *H. calycinum* (Fig. 2). Unlike these morphometric parameters, formation of leaf couples per shoot was significantly influenced only for the hypericin non-producing *H. calycinum*. Hence leaf compactness (IC) was always higher for the MS vitamins supplementation for all three *Hypericum* species (Table 1). The accumulation of fresh weight (FW/DW ratio) did not show a significant difference between media variants for
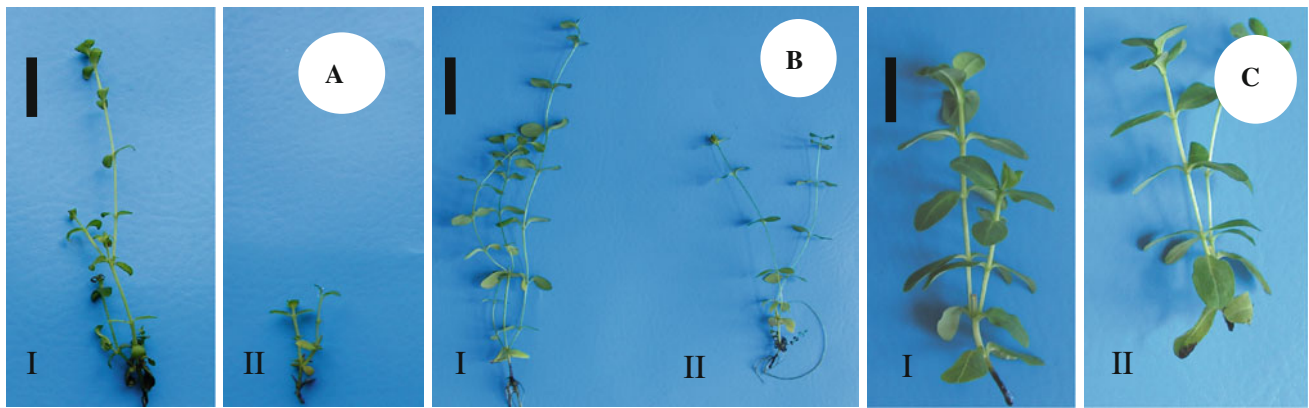
Fig. 1 Shoot cultures of **a** *H. rumeliacum*, **b** *H. tetrapterum* and **c** *H. calycinum* in media I (Gamborg vitamins) and II (MS vitamins). *Scale bar = 1 cm*
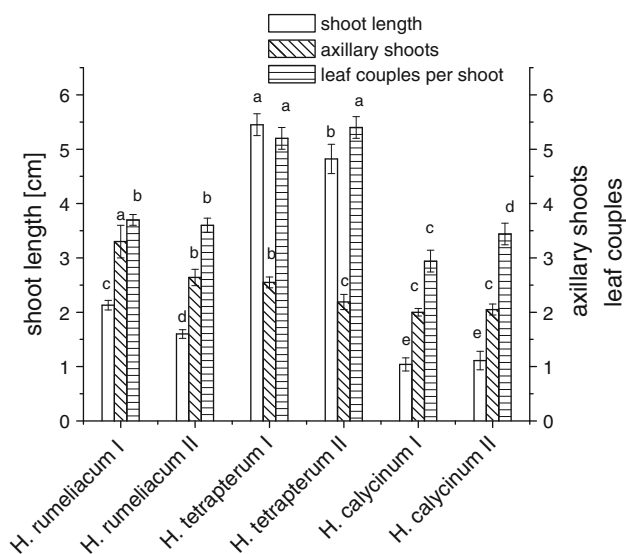
Fig. 2 Influence of vitamin supplementation on the growth parameters of the three *Hypericum* species in media I (Gamborg vitamins) and II (MS vitamins). Data are presented as the mean ± SE. *Different letters above columns indicate significant differences at $P \leq 0.05$*

Table 1 FW/DW ratio and index of compactness of the three *Hypericum* species in media I (Gamborg vitamins) and II (MS vitamins)

Plant species/ medium variant	FW/DW ratio	IC
H. rumeliacum I	4.8 ± 0.16a	1.73d
H. rumeliacum II	5.02 ± 0.2a	2.25c
H. tetrapterum I	4.71 ± 0.15a	0.95f
H. tetrapterum II	4.97 ± 0.12a	1.13e
H. calycinum I	4.75 ± 0.17a	2.83b
H. calycinum II	4.73 ± 0.13a	3.1a

Data are presented as the mean ± SE. Means with the same letters are not significantly different at $P \leq 0.05$

each species and not even when comparing between the different species (Table 1).

The above observations of the impact of Gamborg vitamins formula are fully in agreement with the physiological role of vitamins B in the plant organism reported in literature. As it is known pyridoxine is a co-enzyme in the transamination reactions. Nicotinic acid (in its nicotinamide form) is a component of coenzymes NAD (nicotinamide adeninedinucleotide) and NADP (nicotinamide adeninedinucleotide phosphate). Thiamine occurs in animals, plants, and microbes both as free thiamine and its phosphorylated forms thiamine monophosphate (TMP), thiamine pyrophosphate (TPP), and thiamine triphosphate. These forms act as coenzymes in numerous physiological processes, including glycolysis, the pentose phosphate pathway, and the synthesis of nucleic acids and the niacin-containing coenzyme NADPH (Ahn et al. 2005). Exogenous vitamin B_1 supplementation has been experimentally shown to stimulate the growth of the plant organism. Thus, in 1965 Linsmaier and Skoog (0.4 mg l^{-1} thiamine HCl) revised the Murashige and Skoog's culture medium (0.1 mg l^{-1} thiamine HCl) and established the vital importance of vitamin B_1 for the plant organism. Further on, Gamborg's culture medium (10 mg l^{-1} thiamine HCl) was introduced for the first time in 1968 for the stimulation of the growth of soybean cell culture.

Enhancement of growth parameters has been utilized in practice for the improvement of plant quality in vitro. For instance, Huang et al. (2011) achieved reduction of hyperhidricity of the regenerated shoots of transgenic watermelon phenotypes through increased thiamine hydrochloride supplementation to the basic MS salts formula. Our findings are consistent with these previous reports on the improvement of growth parameters and plant quality achieved by increased vitamin B supplementation in vitro. However, further research is needed to explain the observed lower growth response of *H. calycinum* to enhanced vitamin stimulation, and whether this is an isolated genotypically dependent

effect for this species, or the lack/presence of hypericins could possibly be involved in growth regulation of *Hypericum* in vitro.

Secondary metabolite profiles of in situ collected plant material

Estimation of the amounts of secondary metabolites of the samples of the wild accessions of the three species (Fig. 3) was performed in order to assess possible alterations of the metabolic profile due to the further in vitro development. It was established that while the wild growing *H. tetrapterum* and *H. rumeliacum* had commensurable amounts of total phenolic and flavonoid compounds, the hypericin non-producing *H. calycinum* showed significantly higher quantities of these metabolites on a total assay level (Fig. 3a, b).

Total hypericins (tHyp) amounts in situ were in commensurable levels—0.231 and 0.206 mg g DW^{-1} for *H. rumeliacum* and *H. tetrapterum* respectively (Fig. 3c).

However, *H. rumeliacum* displayed a higher Hyp/psHyp ratio as compared with *H. tetrapterum* (1.07 and 0.31 respectively).

Secondary metabolite profiles of in vitro cultured plants

Phenolic and flavonoid levels in vitro were generally decreased (Table 2) for all three species in comparison with the in situ samples. Nevertheless, it was found that hypericin lacking *H. calycinum* displayed significantly higher amounts of phenolics and flavonoids in comparison with the other two species for both in situ and in vitro conditions. It was also found that samples grown in medium I (Gamborg's vitamins) always displayed decreased levels of both phenolic and flavonoid compounds as compared to the MS vitamins supplementation. As was discussed above, Gamborg vitamins caused improved in vitro multiplication and decrease of leaf compactness of the obtained extractable shoot biomass (Figs. 1, 2; Table 1). On the contrary, plant material derived from medium with
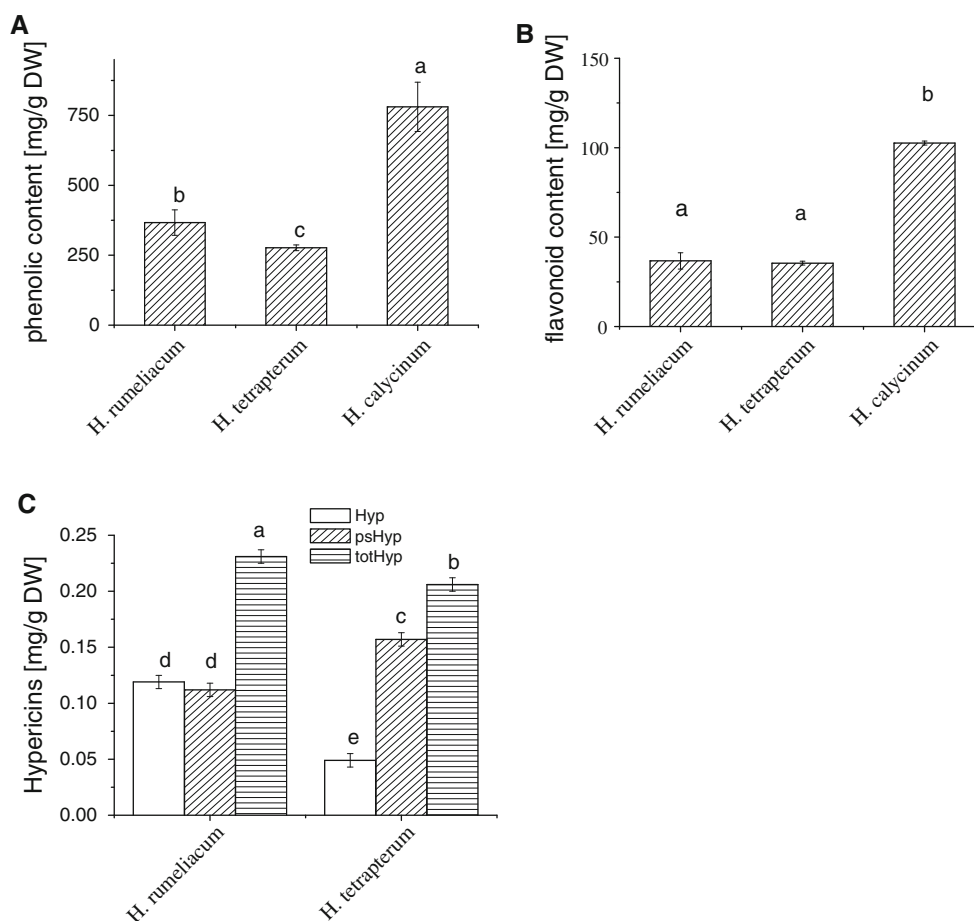
Fig. 3 Polyphenolic compounds in the *in situ* collected material of the three species: **A** total phenolic content, **B** total flavonoid content, **C** hypericin (Hyp), pseudohypericin (psHyp) and total hypericins (totHyp) in accessions of the wild growing *H. rumeliacum, H.* *tetrapterum* and *H. calycinum.* Data are presented as the mean ± SE. *Different letters* above columns indicate significant differences at $P \leq 0.05$

Table 2 Influence of vitamin supplementation on the total phenolic and flavonoid compounds in *H. rumeliacum*, *H. tetrapterum* and *H. calycinum* in media I (Gamborg vitamins) and II (MS vitamins)

Hypericum species/ culture medium	Total phenolics content (mg/g DW)	Total flavonoids content (mg/g DW)
H. rumeliacum I	226.1 ± 0.7d	27.95 ± 0.7d
H. rumeliacum II	250.4 ± 1.6c	30.75 ± 0.8c
H. tetrapterum I	200.54 ± 7.04e	28.04 ± 0.9d
H. tetrapterum II	220.87 ± 5.2d	31.73 ± 0.5c
H. calycinum I	373.59 ± 13.2b	47.76 ± 1.7b
H. calycinum II	449.93 ± 8.4a	55.81 ± 0.6a

Data are presented as the mean ± SE. Means with the same letters are not significantly different at $P \leq 0.05$

MS vitamins supplementation was characterized with depressed growth and compact shoots, with prevailing leaf tissue for all three *Hypericum* species in vitro (Table 1). Therefore the increased phenolic and flavonoid levels in medium II in all three *Hypericum* species might be due to the predominance of leaf tissue in the latter medium. This assumption is also supported by the strong correlation between IC and total phenolic compounds ($r = 0.91$) and IC and flavonoid compounds ($r = 0.85$) for the three *Hypericum* species in the two studied media.

The obtained results support our previous research on *H. rumeliacum* shoots, where benzyl adenine supplementation led to intensive multiplication and biomass gain, but was also accompanied by reduced leaf compactness and a significant drop in phenolic and flavonoid compounds in comparison with the growth regulators-free control (Danova et al. 2010). Hence, different approaches for modification of the morphological development of shoot biomass in vitro could actually be determinative for targeting the secondary metabolite yields in *Hypericum*.

Unlike the phenolic and flavonoid compounds, G5 vitamins increased not only the levels of Hyp and psHyp, but also the Hyp/psHyp ratio for both *H. rumeliacum* and *H. tetrapterum* in comparison with MS vitamins (Fig. 4). Noteworthy, in *H. rumeliacum* Gamborg's vitamins resulted in yields of 0.29 mg g DW^{-1} for hypericin and 1.2 mg g DW^{-1} for pseudohypericin—significantly exceeding the ones of its in situ samples, as well as the yields of *H. tetrapterum* shoots in the present work and also in other literature reports on *Hypericum* productivity in vitro (Kirakosyan et al. 2000; Sirvent and Gibson 2002; Bertoli et al. 2008). Having in mind the comparison with literature data, and the observation that *H. tetrapterum* napthodianthrone levels were not so effectively influenced by in vitro culture as *H. rumeliacum*, these experimental results might be explained by the genetically determined higher biosynthetic capacity of the latter species. As already discussed, infragenetic research performed by
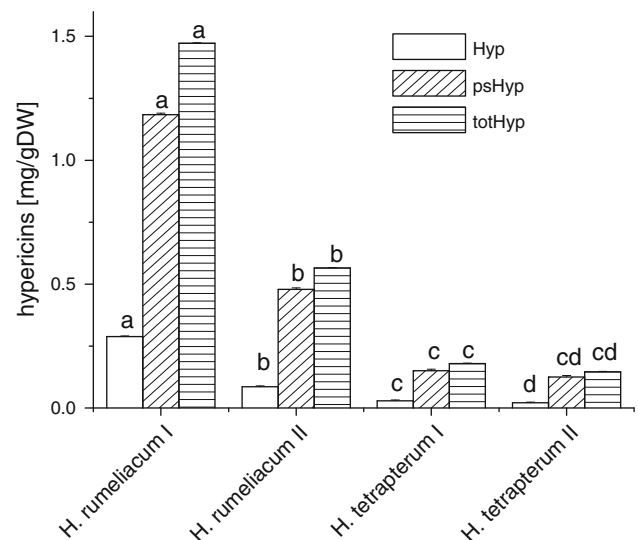
Fig. 4 Influence of vitamin supplementation on hypericin (Hyp), pseudohypericin (psHyp) and total hypericins (totHyp) amounts in shoot cultures of *H. rumeliacum* and *H. tetrapterum* in media I (Gamborg vitamins) and II (MS vitamins). Data are presented as the mean ± SE. *Different letters* above columns indicate significant differences at $P \leq 0.05$

different authors has already proven the higher hypericins biosynthetic capacity of the evolutionary developed *Drosocarpium* section of the *Hypericum* genus (Kitanov 2001; Smelcerovic et al. 2006). However, most probably due to the advantages of the abundant biomass formation in situ, as well as due to its worldwide distribution, *H. perforatum* has gained traditional recognition as the main source of supply of the active ingredients of the complex *Herba Hyperici* extract. Our experimental results indicate the flexibility of hypericin biosynthetic ability of *H. rumeliacum* in vitro and its responsiveness to tissue culture development and modification of growth parameters in vitro. Such observation is in agreement with the theory of Piovan et al. (2004) that namely the ability to carry out the biosynthetic pathway leading to the naphthodianthrone compounds, rather than the absolute amounts produced, should be regarded as a chemical marker of the phylogenetically more advanced sections of genus *Hypericum*.

The present results corroborate with other reports on the effect of enhanced vitamin supplementation on the secondary metabolites production. Thus, in a recent work, a set of culture media compositions were experimented regarding their impact on the growth parameters and secondary metabolites biosynthesis of *Digitalis davisiana* shoot cultures (Gurel et al. 2011). The authors established that LS and Gamborg media were superior in terms of shoots and secondary metabolites production. Similarly, Quintero-Jiménez et al. (2010) found that Gamborg's medium was superior to MS in terms of organogenic shoot formation and whole plant regeneration of *Phaseolus*

vulgaris. Combination of MS salts and Gamborg's vitamins and iron was applied by Jacob and Malpathak (2005) on *Solanum khasianum* hairy root cultures, resulting in enhancement of growth and solasodine production.

In conclusion, our results confirm the importance of the chemotaxonomic approach for selecting a plant object for biotechnological development when targeting the secondary metabolites yields in vitro. Hence, *H. rumeliacum*, belonging to the *Drosocarpium* section is a prospective candidate for hypericin production in controlled in vitro conditions.

Impact of vitamin supplementation on the dark glands of *H. rumeliacum* and *H. tetrapterum*

For both *H. rumeliacum* and *H. tetrapterum* a clear difference in the number of the dark glands per leaf (HGN) was established when comparing the basal, medium and apical shoot samples (Figs. 5, 6; Table 3). The leaves of the basal segments were always with a lower HGN in comparison with the medium and apical ones (Table 3). This effect was not significantly influenced by vitamin supplementation in either of the two species.
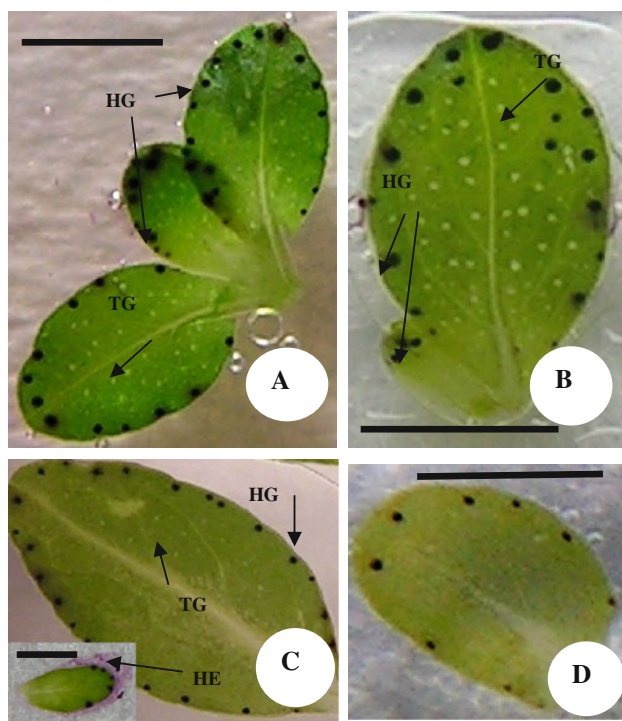

Fig. 5 Photographs of *H. rumeliacum* leaves in media I (Gamborg vitamins) and II (MS vitamins). **a** *H. rumeliacum* apical leaf with visible translucent glands (TG) and hypericum glands (HG), visible on developing leaf primordia in medium I. **b**. Apical leaves of *H. rumeliacum* in medium II. **c** Medium leaf samples of *H. rumeliacum* in medium I, *HE* hypericin exudates leaking out in response to mechanical pressure on the leaf. **d** Basal leaf of *H. rumeliacum* on medium variant I. *Scale bar* = 1 mm

Hypericin dark glands and their possible role in the biogenesis and/or storage of condensed napthodianthrones is a widely discussed topic in literature.

According to Kornfeld et al. (2007) the living cells involved in these structures initially act as a secretory tissue, then lose their functionality and become dead storage areas for secreted phenolic substances. At the end of the reproductive stage, they shrink and lose their shape. Further, another study has shown that the hypericin gland number increases during ontogeny (Zobayed et al. 2006). Therefore, it could be hypothesized that the lower number of hypericin glands in the basal samples in our experiment might be due to the gradual loss of their functionality and shape or to the highest ontogenic age of the basal segment in the individual development of the shoot.

A large number of works have demonstrated the relation between dark glands and hypericins accumulation (Briskin and Gawienowski 2001; Onelli et al. 2002; Piovan et al. 2004). Recently experimental evidence was provided that hypericins are synthesized and accumulated in dark glands (Zobayed et al. 2006). An important finding in the cited study was that when hypericin was measured in isolated glands, no significant difference was observed in the leaf, stem or flower tissues. Also the authors found that the dark glands isolated from the leaves of 3-week-old or 8-week-old plants had similar hypericin concentrations. We should keep in mind the substantial differences in the hypericin amounts between media I and II compositions and moreover between *H. rumeliacum* and *H. tetrapterum*, established in the present work. Thus, it was surprising that the number of dark glands per leaf (HGN for the respective stem segment) did not vary significantly neither between media I and II for each species nor even between *H. rumeliacum* and *H. tetrapterum* (Figs. 5, 6; Table 3). This observation agrees with previous findings that the number of dark glands is seemingly predetermined at the time secretory cells differentiate in the meristem and is considered fixed (Esau 1977). Further, convincing evidence has been provided by Kirakosyan et al. (2003) that in vitro the number of dark glands per leaf is approximately the same for lines differing significantly in hypericin content.

In an attempt to explain this observation, we have also estimated the dark glands density per square millimeter for each leaf of the basal, medium and apical shoot segment in medium I and medium II (Table 3). Our findings indicate that this parameter (HGD), unlike the absolute number of the glands per leaf (HGN), correlates strongly with the hypericins amounts when comparing the two media variants. Thus, for each of the respective segments HGD was higher in medium I when compared to medium II. Also, the density of dark glands of *H. rumeliacum* for all three stem segments was from 2.7 up to 6.8 times higher than the respective ones of *H. tetrapterum* (Table 3). In conclusion, our findings indicate
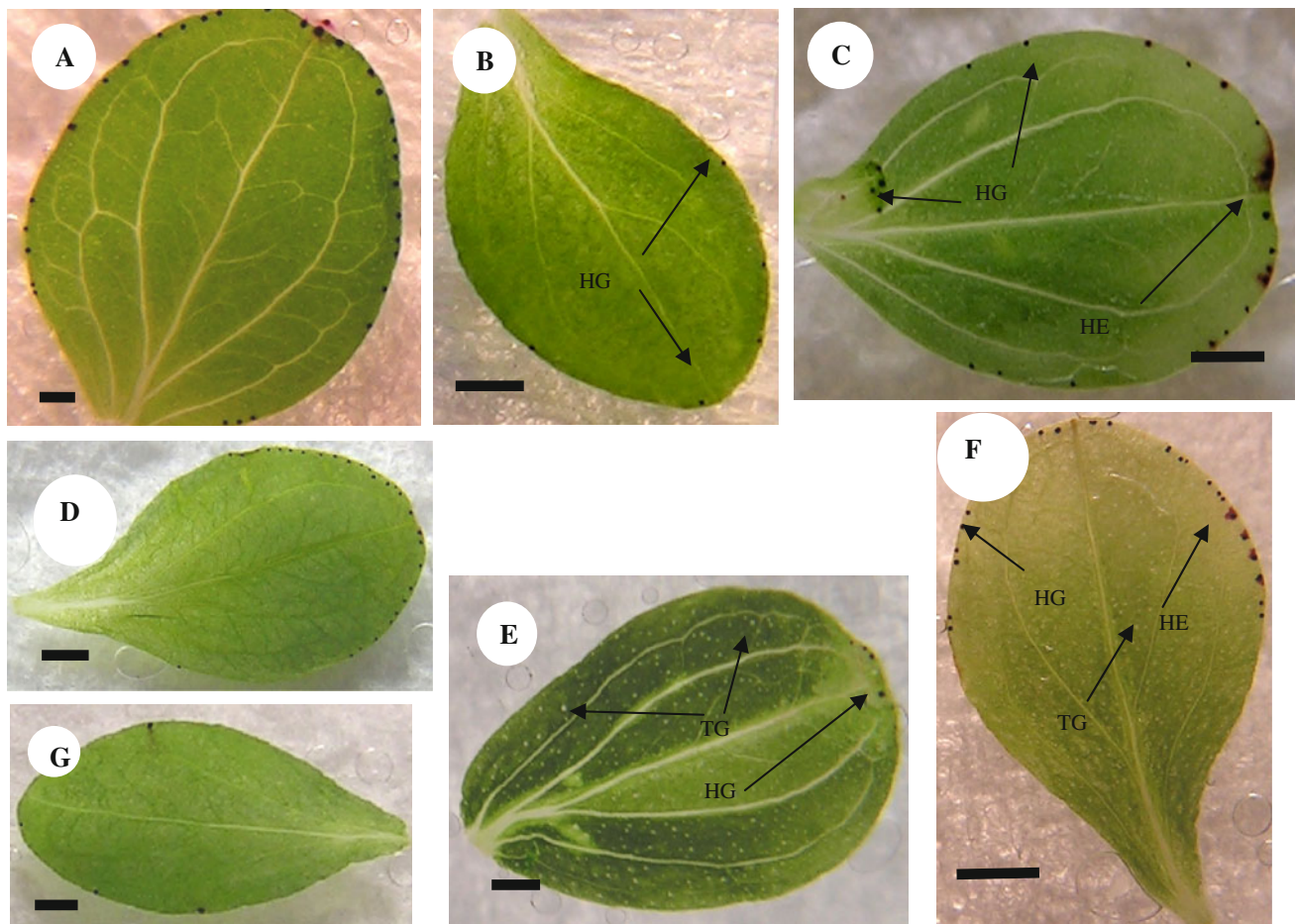
Fig. 6 *Hypericum tetrapterum* leaf samples in media I (Gamborg vitamins) and II (MS vitamins). **a** *H. tetrapterum* apical leaf in medium (I) with a high absolute number of dark glands per leaf (HGN). **b** *H. tetrapterum* apical leaf in medium (I) with a low HGN. **c** *H. tetrapterum* apical leaf in medium (II). HG visible on leaf primordia. **d** Medium leaf of *H. tetrapterum* in medium (I) with high number of hypericin glands. **e** Medium leaf of *H. tetrapterum* in medium (II) with a low number of hypericum glands. **f** Basal leaf of *H. tetrapterum* in medium (I) with high number of hypericin glands on the leaf margins. **g** Basal leaf of *H. tetrapterum* in medium (I) with low number of hypericin glands on the leaf margins. *Scale bar* = 1 mm

that vitamins modification did not alter the formation of dark glands per leaf of the studied *Hypericum* species. In addition, unexpectedly, the number of dark glands per leaf did not vary significantly even when comparing the respective stem segments of the two different *Hypericum* species (which displayed marked differences in hypericins content). However, the morphological changes caused by the increased G5 vitamins supplementation affected substantially the dark glands density, demonstrating a marked relation to the hypericins content in the obtained plant material (r ranging from 0.87 to 0.91 regarding the relation between hypericins content and HGD of leaf samples of the three different stem segments of the two hypericin producing species). Morphological data representation of the hypericin glands per unit of leaf area has also been utilized in other previous works for *H. perforatum* shoot cultures, demonstrating the close relationship between dark gland density and hypericin content (Çirak et al. 2006; Zobayed et al. 2006).

In conclusion, HGD could effectively be used as a rapid diagnostic marker for selection of the experimental conditions for optimization of hypericin yields in *Hypericum* shoot cultures. However, this parameter has to be estimated for shoot samples of the same ontogenic age.

It is also noteworthy to highlight the observation that vitamins B modification altered differentially the studied polyphenolic compounds of the complex *Hypericum* extract—whereas Gamborg's vitamin supplementation raised hypericins amounts, it also led to a decrease in the total phenolic and flavonoid contents. This effect was shown to be connected with the different morphological basis of the production and deposition of the secondary metabolites present in *Herba Hyperici*. The depressed growth and hence the increased compactness of the obtained extractable biomass in MS medium led to increased phenolic and flavonoid compound levels in all three *Hypericum* species (both hypericin producing and

Table 3 Morphometric characteristics of hypericin glands number per leaf (HGN), leaf area (LA [mm^2]) and hypericin glands density (HGD = HGN/mm^2) for *H. rumeliacum* and *H. tetrapterum* medium I (Gamborg vitamins) and medium II (MS vitamins)

Plant species/culture medium	Basal leaf samples			Medium leaf samples			Apical leaf samples		
	HGN	LA (mm^2)	HGD	HGN	LA (mm^2)	HGD	HGN	LA (mm^2)	HGD
H. rumeliacum I	7.67 ± 0.52a	1.48 ± 0.21c	7.84 ± 0.72a	16.19 ± 0.65a	4.45 ± 0.41c	5.56 ± 0.5a	12.02 ± 0.72a	2.26 ± 0.29b	7.28 ± 0.62a
H. rumeliacum II	6.35 ± 0.47b	1.49 ± 0.2c	5.39 ± 0.32b	13.18 ± 0.56a	4.03 ± 0.39c	4.86 ± 0.28a	12.12 ± 0.57a	2.43 ± 0.18b	5.2 ± 0.3b
H. tetrapterum I	3.34 ± 0.7c	4.46 ± 0.8b	1.14 ± 0.23c	12.66 ± 1.5a	16.18 ± 1.97b	0.88 ± 0.06b	13.75 ± 1.16a	7.37 ± 0.8a	2.5 ± 0.26c
H. tetrapterum II	4.93 ± 0.9c	6.26 ± 0.9a	0.98 ± 0.09c	14.63 ± 1.45a	23.15 ± 2.27a	0.72 ± 0.05b	13.4 ± 1.4a	8.1 ± 0.8a	1.91 ± 0.17d

Data are presented as the mean ± SE. Means with the same letters are not significantly different at $P \leq 0.05$

hypericin non-producing). On the other hand, obtaining shoots with prevailing stem tissue, but with increased dark glands density proved to be a tool for increasing hypericin yields in vitro.

MDA and H_2O_2 content

The levels of the studied stress markers—MDA and H_2O_2, were markedly higher in *H. rumeliacum* in comparison with *H. tetrapterum* and *H. calycinum* (Fig. 7). Also, for the two hypericin producing species we established a positive relation between hypericins content and the levels of these two parameters in media I and II. Thus, regarding MDA content correlations were $r = 0.87$ and $r = 0.92$ for hypericin and pseudohypericin, respectively. For H_2O_2 we obtained $r = 0.91$ (hypericin) and $r = 0.95$ (pseudohypericin). Noteworthy, the MDA and H_2O_2 levels for the hypericin-non producing *H. calycinum* were not only considerably lower than of the other two, but also did not seem to differ significantly between media I and II (Fig. 7). Thus, our results indicate a strong correlation between high hypericin and pseudohypericin productivity and oxidative stress of the shoot cultures of *H. rumeliacum* and *H. tetrapterum*. Obviously, hypericin and pseudohypericin are related to the physiological status of *Hypericum* species in tissue culture conditions. On the other hand a markedly high negative relation between the total phenolic content and H_2O_2 ($r = -0.50$) and MDA ($r = -0.47$), as well as the flavonoid compounds levels and H_2O_2 ($r = -0.62$) and MDA ($r = -0.59$), were also observed.

Plant cell, tissue and organ cultures produce free radicals, lipid peroxides, as well as toxic products of lipid peroxidation (Benson 2000). The antioxidant profiles of in vitro cultured plants vary during different cultivation phases as well, because in vitro manipulations lead to principle metabolic and developmental changes, some of which cause an increased production of free radicals (Benson 2000). Generally, the presence of phenolic compounds in the explant correlates with the elevated levels of oxidative stress in vitro (Benson 2000). In the present work we established, a high positive relation between MDA, H_2O_2 and hypericins amounts, and a high negative relation of the stress markers with the phenolic and flavonoid compounds when comparing the six *Hypericum* samples in the present work. Thus, further research is needed to reveal the possible synergistic or antagonistic relations between the individual components of the complex *Hypericum* extract and assess their impact on the physiological state of the plant organism.

A quite recent research draws insight into this matter by revealing that raised H_2O_2 might actually mediate increased hypericin production as an adaptation stress response in *H. perforatum* cell culture Xu et al. (2011). The
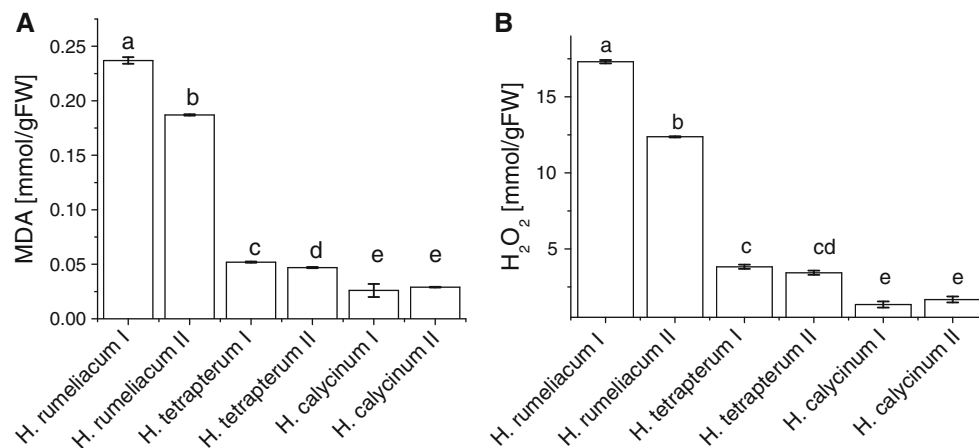
Fig. 7 Influence of vitamin supplementation on: **A** malondialdehyde (MDA) and **B** hydrogen peroxide (H_2O_2) content in *H. rumeliacum*, *H. tetrapterum* and *H. calycinum* media I (Gamborg vitamins) and II (MS vitamins). Data are presented as the mean $\pm$ SE. *Different letters* above columns indicate significant differences at $P \leq 0.05$

process was shown to be mediated through the increased plasma membrane NADPH oxidase activity. According to Yang et al. (2010), modulation of ascorbate peroxidase activity in *Nitraria tangutorum* calli might be associated with a NADPH oxidase—dependent H_2O_2 generation under salinity stress. Other works have also shown that H_2O_2 is responsible for the activation of antioxidant enzymes and of conferring stress tolerance in the plant organism (Tanou et al. 2009; Sun et al. 2010; Hernandez et al. 2010; Zhang et al. 2010, 2011).

In conclusion, we could hypothetically suppose that either the higher metabolic activity leading to hypericin production might be connected with generation of reactive oxygen species in vitro, or the increased oxidative stress could be a consequence of a possible auto-toxicity of high hypericin levels, inherently present in the highly producing species/media variants. Having in mind, that hypericin production is characteristic for the evolutionary more developed sections of the genus, the question might arise whether its production could actually have evolved not only as a repelling phototoxic agent, but also as an adaptive factor in overcoming adverse environmental conditions. Hence, stimulated hydrogen peroxide generation in vitro might also be an adaptative response towards the physiological stress of the tissue culture conditions imposed on the plant organism. In conclusion, the genetically predetermined high hypericin production capacity of certain *Hypericum* species makes them favorable objects for successful biotechnological yield of naphthodianthrones. However, the constitutively high hypericin production in vitro also imposes the necessity of avoiding possible recalcitrance problems due to the elevated oxidative stress in potential upscale of the process.

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